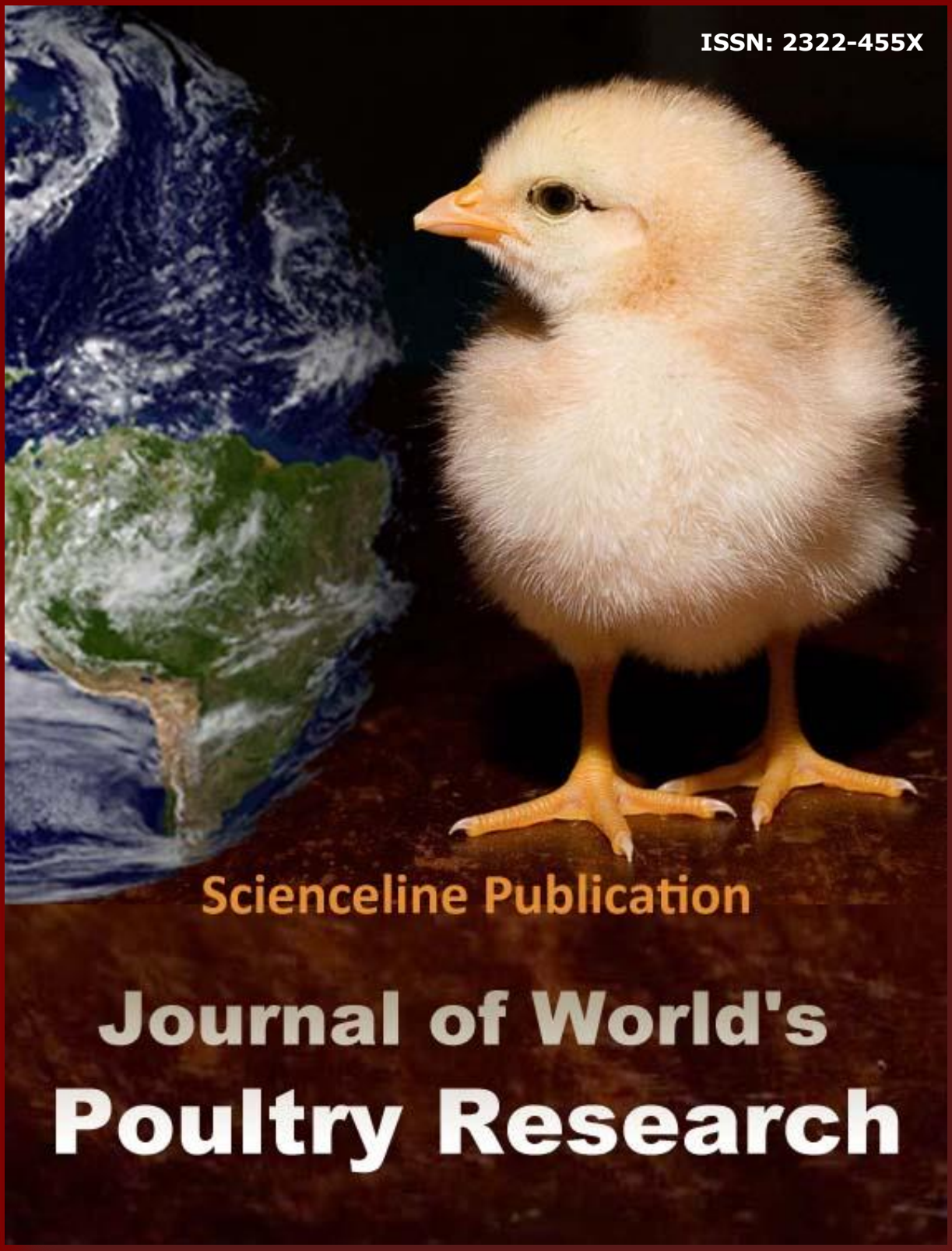
In the Name of God



An international peer-reviewed journal which publishes in electronic format

Volume 10, Issue 4, December 2020

Journal of World's Poultry Research

J. World Poult. Res. 10 (4): 545-661; December 25, 2020

Editorial Team

Advisory Board

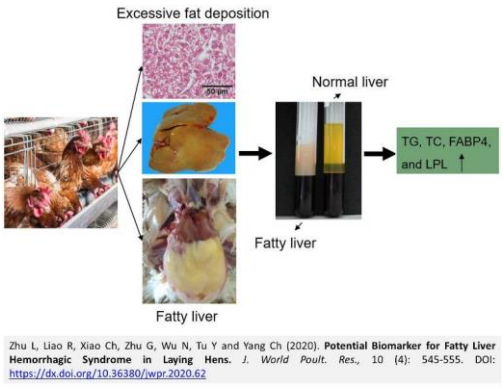
Volume 10 (4); December 25, 2020

Research Paper

Potential Biomarker for Fatty Liver Hemorrhagic Syndrome in Laying Hens.

Zhu L, Liao R, Xiao Ch, Zhu G, Wu N, Tu Y and Yang Ch.

J. World Poult. Res. 10(4): 545-555, 2020; pii: S2322455X2000062-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.62



Zhu L, Liao R, Xiao Ch, Zhu G, Wu N, Tu Y and Yang Ch (2020). **Potential Biomarker for Fatty Liver Hemorrhagic Syndrome in Laying Hens.** *J. World Poult. Res.*, 10 (4): 545-555. DOI: https://dx.doi.org/10.36380/jwpr.2020.62

ABSTRACT: Fatty liver hemorrhagic syndrome is more common in laying hens with excess body weight (BW) and in the middle and late phase of egg production. However, no specific biomarkers in chickens can be used to diagnose liver steatosis or liver injury. The present study aimed to assess whether BW can be used to predict fatty liver in aged laying hens. This study also searched for potential plasma FLHS biomarkers. For these purposes, correlation among BW, relative weight of liver and abdominal fat, and plasma markers were analyzed in Hy-line brown laying hens. Furthermore, plasma levels of potential biomarkers were analyzed during the formation of fatty liver. Concentrations of triglycerides and total cholesterol were positively associated with BW in aged laying hens, while liver fat deposition was similar among chickens with different BW, indicating that BW cannot be used as the only criterion to discriminate aged laying hens with liver steatosis. A trend of increasing triglyceride, total cholesterol, fatty acid-binding protein 4 (FABP4), and lipoprotein lipase levels was found as age increased, and they were positively associated with BW indicating that they might be risk markers for FLHS in laying hens. The findings indicated that the plasma level of FABP4 was positively associated with the severity of fatty liver in aged laying hens. All the above results suggested that FABP4 might be a potential diagnostic indicator for FLHS.
Key words: Biomarker, Egg production, Fatty liver, Laying hens, Poultry

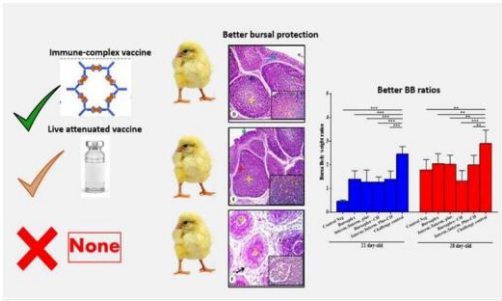
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Immune-Complex Infectious Bursal Disease Virus versus Live Attenuated Vaccines to Protect SPF Chicken against Very Virulent Virus Challenge.

Abou El-Fetouh MS, Hafez MH, El-Attar ER and El-Agamy ME.

J. World Poult. Res. 10(4): 556-564, 2020; pii: S2322455X2000063-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.63



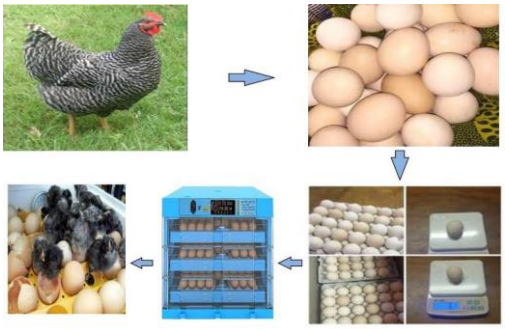
Abou El-Fetouh MS, Hafez MH, El-Attar ER and El-Agamy ME (2020). **Immune-Complex Infectious Bursal Disease Virus versus Live Attenuated Vaccines to Protect SPF Chicken against Very Virulent Virus Challenge.** *J. World Poult. Res.*, 10 (4): 556-564. DOI: https://dx.doi.org/10.36380/jwpr.2020.63

ABSTRACT: In this study infectious bursal disease (IBD) vaccinations were evaluated against very virulent IBD (vvIBDV) challenge and were compared. A total of 120-day-old white Leghorn SPF chickens were divided into 6 groups (each was 20 birds). Two groups were vaccinated on either day 1 with an immune-complex vaccine. The second groups were vaccinated at days 9 and 14 of age using intermediate and intermediate plus IBD vaccines, respectively the balance groups are controls. All vaccines were administered according to the manufacturer's instructions. The challenge was conducted on the 16 days of age using 105 EID50 /0.1 ml of a vvIBDV strain via the oculonasal route. The antibody immune response was monitored in all groups at 14, 21, 28, and 35 days of age. The performance, bursal gross lesions, challenge virus detection, and bursal histopathology were evaluated in vaccinated non challenged and vaccinated challenged birds at days 21 and 28 of age. All vaccinated groups were protected against the vvIBDV challenge compared to 40% mortality in the challenge control group. Both the immune-complex and live attenuated IBD vaccine groups showed similar bursa body weight (BB) ratios compared to the negative control group. The immune-complex vaccinated groups antibody titers were significantly higher except on 28th day of age. Upon challenge, the intermediate/intermediate plus vaccinated challenged group showed higher antibody titers at 21 and 35th with the challenge virus detection and quantification on day 28. The immune-complex vaccinated challenged group developed milder bursal histopathology signs but no differences between the 2 vaccine programs were seen. It can be understandable, the use of either immune-complex vaccine at day-old or early vaccination with intermediate followed by intermediate plus live attenuated IBD vaccines induced protective antibody titers and protect chickens against an early vvIBDV challenge. The suggested schedules need further evaluation in commercial broilers with maternal derived IBD antibodies to simulate field conditions.
Keywords: Immune-Complex vccine, Infectious Bursal Disease, Live Attenuated Vaccine, SPF Chicken

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Reproductive Performance of Koekoek Chickens at Different Levels of Feed Restrictions.



Molapo Motsoene S, Webb E, Aloycia Mahlehla M, Chabeli Th, and Kompi P.

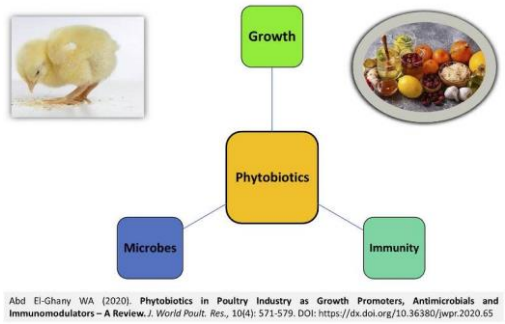
J. World Poult. Res. 10(4): 565-570, 2020; pii: S2322455X2000064-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.64

ABSTRACT: The objective of the present study was to determine the impact of the feeding levels on the reproductive characteristics of Koekoek chickens. A total of 270 Koekoek chickens were randomly assigned to 4 feeding level treatments in a completely randomized design. The four feeding level treatments were fully fed during the rearing and laying phase (AA), fully fed during the rearing phase and restricted feeding during the laying phase (AR), restricted feeding during the rearing phase and fully fed during the laying phase (RA), and restricted both during the rearing and laying phase (RR). The General Linear Model procedure (SPSS software, version 17) was used to analyze the data set. The pubic bone measurements were 23.6, 25.1, 16.1, and 15.1 mm for chickens that received AA, AR, RA, and RR treatments, respectively, at 18 weeks of age. At 32 weeks of age, chickens given AA and RA treatments had wider pubic bones than chickens given AR and RR treatments. Combined ova and oviduct weights were higher in the fully fed chickens at 18 weeks of age. Koekoek chickens in AA treatment had the highest average egg production. Chickens given AR treatment had lower average egg weights than those given AA, AR, and RR treatments. Chickens treated with AA and AR reached puberty earlier than those that were treated with the RA and RR treatments. The eggs produced by chickens given RR treatment had a higher average hatching percentage. The lowest percentage of hatches was observed in chickens that were fed ad libitum during the rearing phase. In conclusion, the feed restriction only during the rearing phase improved the reproduction performance of Koekoek chickens.
Keywords: Egg weight, Fully fed, Hatchability, Koekoek, Laying percentage, Oviduct, Pubic bone, Restricted

[Full text-PDF] [XML] [Crossref Metadata]

Review

Phytobiotics in Poultry Industry as Growth Promoters, Antimicrobials and Immunomodulators – A Review.



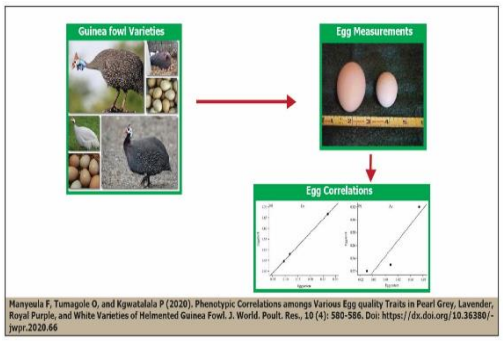
Abd El-Ghany WA.
J. World Poult. Res. 10(4): 571-579, 2020; pii: S2322455X2000065-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.65

ABSTRACT: Due to the hazardous use of antimicrobials in poultry production sector, development of drug resistance have become a worldwide problem. Therefore, using biotic or natural products, such as phytobiotics (phytogenics or botanicals) have received a great attention as antibiotic substitutes. The use of phytobiotics or their constituents have been considered as a relatively new class of natural herbs that gained popularity and acceptability among poultry farmers. The incorporation of several types of phytobiotic additives in poultry feed have proved their ability to enhance the productive performance of broilers as well as layers. Moreover, phytbiotics presented great efficacy in counteracting intestinal pathogenic microorganism while maintaining the population of normal inhabitant beneficial microflora. Immunostimulatory effect on both humoral and cellular immunity as well as antioxidant properties were recorded as characters of phytobiotics. Therefore, this review article aimed to give a spotlight on the uses of different types of phytobiotics as poultry dietary additives to improve the productive parameters, reduce the pathogenic intestinal bacteria, and potentiate the immune response, especially after vaccination processes.
Keywords: Antimicrobial, Immunity, Performance, Plants, Poultry

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Phenotypic Correlations among Various Egg Quality Traits in Pearl Grey, Lavender, Royal Purple, and White Varieties of Helmeted Guinea Fowl.



Manyeula F, Tumagole O and Kgwatalala P.

J. World Poult. Res. 10(4): 580-586, 2020; pii: S2322455X2000066-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.66

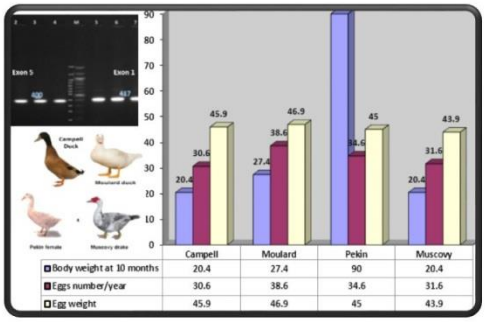
ABSTRACT: Guinea fowls are increasingly popular in Botswana since

they not only provide an alternative to access protein in the form of eggs and meat but also become a good source of income for the peasants. There are different varieties of Guinea fowl in Botswana, including pearl grey, lavender, royal purple, and white. Indeed, there is a need to conduct more studies related to the phenotypic correlations among egg quality traits in different varieties of helmeted guinea fowl found in Botswana. Therefore, the present study was targeted toward the evaluation of both the external and internal quality characteristics of the four different varieties of the domesticated helmeted guinea fowl found in Botswana. In this regard, a total of 150 eggs were collected from the so-called varieties of domesticated helmeted guinea fowl. The egg weight was positively and significantly correlated with egg length, egg width, shell weight, egg surface area, and egg volume; however, the egg weight was negatively correlated with egg shape index. Of the four varieties of domesticated helmeted guinea fowl found in Botswana, the white variety had the strongest correlation coefficients with various external egg quality traits and different internal egg quality characteristics. The lavender variety had the highest correlation coefficients with internal and external egg quality traits. It seems that the selection for higher egg weight as is the case in the current egg grading system can lead to the greatest improvements in other egg quality characteristics in the white and lavender varieties, compared to the pearl grey and royal purple varieties. Therefore, the lavender and white varieties are the potential candidates for the possible selection of layer-type guinea fowl varieties.
Keywords: Botswana, Egg traits, Guinea fowl, Layer-type

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Polymorphism of the Prolactin Gene in Egyptian Duck Breeds.



Sabry NM, Mabrouk DM, Abdelhafez MA, El-Komy EM and Mahrous KF.

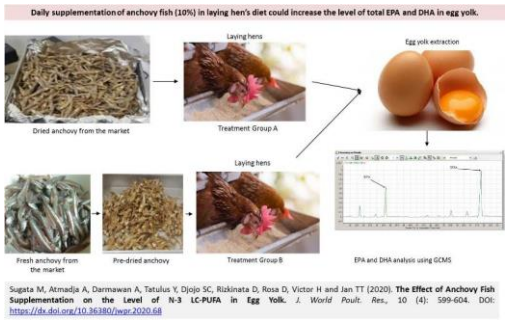
J. World Poult. Res. 10(4): 587-598, 2020; pii: S2322455X2000067-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.67

ABSTRACT: In avian, the prolactin hormone triggers and regulates ovarian follicle development. This study aims to detect the Prolactin (*PRL*) gene polymorphisms (exons 1and5) in four Egyptian duck breeds, namely Campbell, Moulard, Muscovy, and Pekin using PCR-RFLP technique and sequence analysis. It also investigated the association of this gene with egg production, egg weight, and body weight. The present results revealed that *PRL* gene exon 1 and part of intron 1 showed two alleles A and B (polymorphic) in each of Campbell and Moulard, however, Muscovy and Pekin had only one allele (monomorphic). The allele A was more dominant with frequencies of 0.70, 0.60, and 1.00, compared to the allele B (0.30, 0.40, and 0.00) for Campbell, Moulard, and Muscovy, respectively. For Pekin, the allele B only appeared with the frequency of 1.0. Ducks with the high frequency of allele A were superior at egg weight, compared to others. Furthermore, for *PRL* gene exon 5, there were two alleles G and C (polymorphic) in Campbell, Moulard, and Muscovy, however, Pekin had only one allele (monomorphic). The allele G was more dominant (0.15, 0.74, 0.0, and 0.84) than the allele C (0.85, 0.26, 1.0, and 0.15) for Campbell, Moulard, Pekin, and Muscovy, respectively. Ducks having a high frequency of allele C were superior at egg production. Furthermore, there were many single nucleotide polymorphisms (SNPs) in the sequences in all breeds. The utmost ones exist at the restriction sites of *Xba*I enzyme for the amplified fragment, in the promotor, exon 1 and intron 1 (T378C in intron 1), and *Dra*I enzyme for that in exon 5 (A5871G in exon 5).
Keywords: Duck, Genetic polymorphism, Genotyping, Prolactin gene

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

The Effect of Anchovy Fish Supplementation on the Level of N-3 LC-PUFA in Egg Yolk.



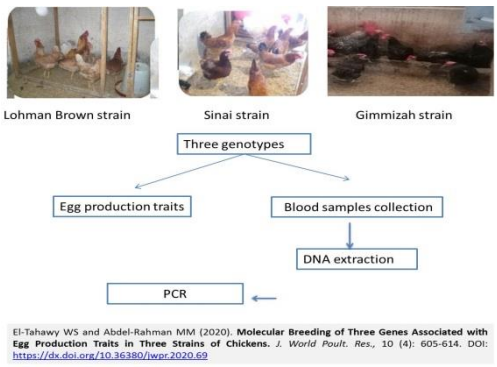
Sugata M, Atmadja A, Darmawan A, Tatulus Y, Djojo SC, Rizkinata D, Rosa D, Victor H and Jan TT.

J. World Poult. Res. 10(4): 599-604, 2020; pii: S2322455X2000068-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.68

ABSTRACT: Since the recommended daily intake of n-3 LC-PUFA is rarely met, interest in food enrichment has been increasing. It is known that dietary supplementation could alter the level and type of PUFA in the egg. Hence, the present study focused on the enrichment of egg yolk by the addition of 10% anchovy fish to the chicken diet. Based on gas chromatography analysis, dried and pre-dried anchovy from Indonesia contained a considerable amount of total eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which was 60.10 g and 68.80 mg/100 g, respectively. After 24 days of anchovy supplementation, DHA-rich anchovy fish oil diet caused a significant increase of DHA but not EPA in egg yolk. Hens fed with anchovy could produce eggs with a higher amount of total EPA and DHA, which was up to 155.98-201.53%, as compared to control eggs. Furthermore, the sensory profile of control and enriched eggs was also evaluated. There was no significant difference in texture, aroma, flavor, and appearance between control and enriched eggs. In conclusion, this study indicated that anchovy fish supplementation could increase the level of EPA and DHA in egg yolk without causing any sensory changes in the yolk.
Keywords: Anchovy, DHA, Egg yolk, Enrichment, EPA
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Molecular Breeding of Three Genes Associated with Egg Production Traits in Three Strains of Chickens.



El-Tahawy WS and Abdel-Rahman MM (2020). **Molecular Breeding of Three Genes Associated with Egg Production Traits in Three Strains of Chickens.** *J. World Poult. Res.*, 10 (4): 605-614. DOI: https://dx.doi.org/10.36380/jwpr.2020.69

El-Tahawy WS and Abdel-Rahman MM.

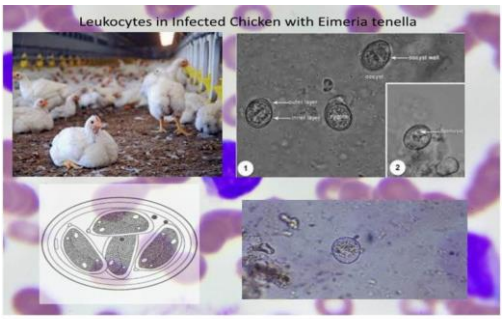
J. World Poult. Res. 10(4): 605-614, 2020; pii: S2322455X2000069-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.69

ABSTRACT: Breeding programs play an important role in increasing the performance of chickens. The poultry industry regards growth and reproduction as the two most economically valued characteristics for providing adequate animal proteins. Genetic variations are the basis of animal breeding. The present study was conducted on three genes, including Prolactin, 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase (HMGCR), and Gonadotropin-Releasing Hormone Receptor (GNRHR). DNA was isolated from 48 chickens taken from three strains Lohmann Brown (17), Sinai (24), and Gimmizah (7) for Prolactin, HMGCR, and GNRHR gene amplification by using the PCR protocol. Electrophoresis was performed on the PCR products and the bands were viewed on a transilluminator. The size of the Prolactin gene, HMGCR, and GNRHR were 154, 675, and 210 bp, respectively. For the Sinai strain, five bands for Prolactin, two bands for HMGCR, and six bands for GNRHR were found while for the Lohmann Brown strain, five bands of Prolactin gene, three bands for HMGCR, and five bands for GNRHR were found. Regarding the Gimizah strain, two bands were found for Prolactin and GNRHR genes and there was only one band for the HMGCR gene. The Lohmann Brown strain respectively matured 13 and 91 earlier than Gimizah and Sinai strains with a higher egg number during the first 90 days.
Keywords: Breeding, Chickens, Egg production, GNRHR gene, HMGCR gene, Prolactin gene, PCR

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

The Effect of Dietary Administration of Virgin Coconut oil on Differential Leukocytes in Infected Chicken with Eimeria tenella.



Faradilla ZSh, Yunus M and Hermadi HA (2020). **The Effect of Dietary Administration of Virgin Coconut oil on Differential Leukocytes in Infected Chicken with *Eimeria tenella*.** *J. World Poult. Res.*, 10 (4): 615-622. DOI: https://dx.doi.org/10.36380/jwpr.2020.70

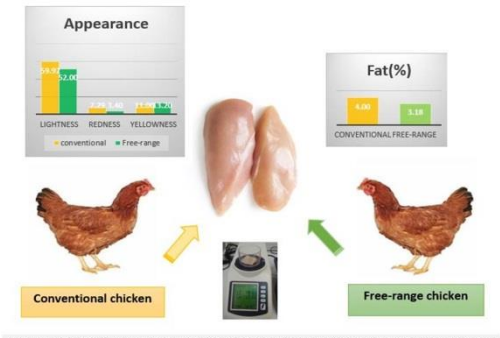
Faradilla ZSh, Yunus M and Hermadi HA.
J. World Poult. Res. 10(4): 615-622, 2020; pii: S2322455X2000070-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.70

ABSTRACT: Coccidiosis is the main problem in poultry diseases. It is caused by the parasite *Eimeria tenella,* which induce the immune response of leukocyte. Anticoccidial drugs are administered in the poultry feed to control coccidiosis. However, taking medication for a long time can lead to resistance. Recent studies have indicated that Virgin Coconut Oil (VCO) has some benefits, including anti-inflammatory effects. The present research aimed to identify the effect of VCO at the different doses in improving the various leukocyte counts of chickens infected with *E. tenella*. Male chickens were divided into five groups (T0, T1, T2, T3, and T4) and treated for 28 days. T0 was neither infected with *E. tenella* nor get treatment, T1 was only infected with *E. tenella*, T2 was treated with 5 ml/kg VCO feed and had *E. tenella* infection, T3 was treated with 10 ml/kg VCO feed and had *E. tenella* infection, and T4 was treated 20ml/kg VCO feed and had *E. tenella* infection. Differential leukocyte was counted with a blood cell counter. The data obtained were analyzed using ANOVA and Duncan's Multiple Range Test. The results indicated that a dose of 10 ml/kg feed and 20ml/kg feed of VCO could improve the differential leukocyte counts of chickens infected with *E. tenella* by maintaining the counts of basophil, eosinophil, heterophil, monocyte, and lymphocyte in the normal range. The present study concluded that VCO by a dose of more than 10 ml/kg would improve the differential leukocyte counts of chickens infected with *E. tenella*.
Keywords: Differential leukocyte count, *Eimeria tenella*, Virgin Coconut Oil

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Characteristics of Carcass Traits and Meat Quality of Broiler Chickens Reared under Conventional and Free-range Systems.



Davoodi P and Ehsani A (2020). **Characteristics of Carcass Traits and Meat Quality of Broiler Chickens Reared under Conventional and Free-range Systems.** *J. World Poult. Res.*, 10 (4): 623-630. DOI: https://dx.doi.org/10.36380/jwpr.2020.71

Davoodi P and Ehsani A.

J. World Poult. Res. 10(4): 623-630, 2020; pii: S2322455X2000071-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.71

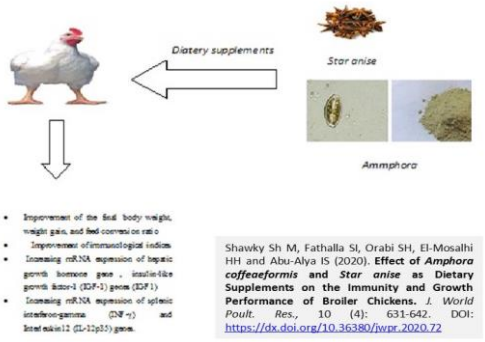
ABSTRACT: Alternative chicken production systems have become popular in recent years due to animal welfare criteria and consumer's

perceptions. General beliefs express that the meat quality of chicken reared under free-range systems is better than that of chickens under conventional production conditions. The aim of this study was to compare the meat quality and carcass traits of chickens raised in conventional and free-range systems. Either conventional or free-range systems used meat-type Hubbard JA57 birds with a slaughter age of approximately 78 days. For assessing carcass traits and meat quality, six male chickens were selected from each system. The meat quality parameters, pH at 45 minutes, ultimate pH, color coordinates, drip loss, cooking loss, and water-holding capacity were measured. Furthermore, proximate parameters, such as crude protein, total fat, and crude ash were determined. There were no significant differences in main carcass yield and breast muscles between chickens reared in two systems, however, color values dramatically were influenced by rearing systems. Breast muscle samples from birds reared under the conventional system had a smaller hue angle and saturation value than those from the free-range birds. Moreover, the drip loss parameter was significantly higher in free-range chickens. The ash and protein contents of breast muscles were similar although raw breast meat from free-range birds had significantly lower fat content. The results prove that a free-range rearing system can modify the appearance, color values, and fat content of chicken meat and it can be a part of the interests of meat production consumers.
Keywords: Broiler chickens, Free-range, Hubbard JA57, Intensive rearing system, Meat quality

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Effect of *Amphora coffeaeformis* and *Star anise* as Dietary Supplements on the Immunity and Growth Performance of Broiler Chickens.



Shawky Sh M, Fathalla SI, Orabi SH, El-Mosalhi HH and Abu-Alya IS.

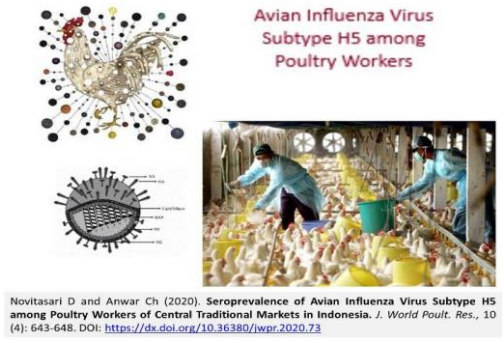
J. World Poult. Res. 10(4): 631-642, 2020; pii: S2322455X2000072-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.72

ABSTRACT: The present study was designed to evaluate the impacts of daily dietssupplemented with *Amphora coffeaeformis* and *Star anise* on growth performance and immunity of Cobb broiler chickens. *Amphora coffeaeformis* is considered a potent free radical scavenger due to the presence of β-carotene and fucoxanthin, which are used widely as food additives. *Star Anise* has a natural antioxidant, which can also be used for the chemo-prevention of disease occurring due to oxidative deterioration. A total of 270 broiler chickens were divided into three groups, each with three replicates of 30 birds. The control group (G1) was given the basal diet, the *Amphora* group (G2) received *Amphora* in a dose of 1g/Kg in the ration, and the *Star anise* group (G3) received *Star anise* in a dose of 2g /Kg in the ration. The results indicated that *Amphora* and *Star anise* significantly improved the final body weight, weight gain, and feed conversion ratio, total white blood cells count, phagocytic activity percentage, phagocytosis index in plasma, IgM, IgG, and A/G ratio in serum. In addition, *Amphora* and *Star anise* significantly increased mRNA expression of hepatic growth hormone gene, insulin-like growth factor-1 (IGF-1) genes (IGF1), and mRNA expression of splenic interferon-gamma (INF-γ) and Interleukin 12 (IL-12p35) genes from broiler chickens, compared to the control group. In conclusion, the use of fed additives, such as *Amphora coffeaeformis*and *Star anise* in the diet of broiler chickens for 35 days was sufficient to improve broiler growth performance and could modulate their immunity.
Keywords: *Amorphacoffeaeformis*, Broiler chickens, Diet supplementation, Growth performance, Immunity, *Star anise*

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Seroprevalence of Avian Influenza Virus Subtype H5 among Poultry Workers of Central Traditional Markets in Indonesia.



Novitasari D and Anwar Ch.

J. World Poult. Res. 10(4): 643-648, 2020; pii: S2322455X2000073-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.73

ABSTRACT: Avian Influenza (AI) has been spread rapidly in almost all the provinces of Indonesia by the end of 2007, and it has become endemic. Avian Influenza viruses can be infecting to human from direct or indirect contact with the infected or dead poultry, and a visit to the wet poultry market in the neighborhood. Seroprevalence studies can be used to identify the clinical key, epidemiological studies, and the spread of AI viruses in humans. The aim of present study was to investigate the seroprevalence of Avian Influenza (AI) virus, subtype H5 among poultry workers at the central traditional market in industrial. To meet the mentioned demand, 26 blood samples were collected from the poultry workers via the median cubital vein. The antibody titer was examined using Hemagglutination Inhibition (HI) assay using H5 antigen from duck licensed under A/Dk/Indonesia/AU-78/12 (H5N1) and three kinds of red blood cells taken from horse, chicken and guinea pig. The serum samples were added with Receptor Destroying Enzyme (RDE) with a ratio of 3:1 (v/v) for an overnight, and pretreated with 10% of red blood cells before the HI assay was conducted. Based on the findings, it can be concluded that the percentage of seroprevalence of Avian Influenza (AI) virus, subtype H5 among poultry workers at central traditional market was 0%. Thirteen samples showed a negative result of the HI test. All of the workers' blood serum obtained less than 24 antibody titer from the HI test. As the results showed, the research on the poultry workers in the traditional market was carried out, and it obtained negative results; all the workers were not infected with the Avian

Influenza virus. In other words, Avian Influenza is not meaningful in poultry farm workers in Sidoarjo suburb traditional farms.
Keywords: Avian Influenza, Poultry workers, Seroprevalence, Traditional market.

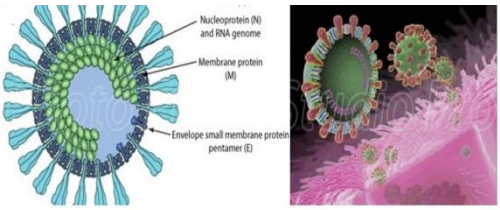
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Characterization and Analysis of the Major Structural Protein Genes of the Recently Isolated Avian Infectious Bronchitis Virus in Egypt.

Yehia N, Said D and Zanaty AM.

J. World Poult. Res. 10(4): 649-661, 2020; pii: S2322455X2000074-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.74



Yehia N, Said D and Zanaty AM (2020). **Characterization and Analysis of the Major Structural Protein Genes of the Recently Isolated Avian Infectious Bronchitis Virus in Egypt.** *J. World Poult. Res.*, 10 (4): 649-661. DOI: https://dx.doi.org/10.36380/jwpr.2020.74

ABSTRACT: Infectious Bronchitis Virus (IBV) is a severe infectious disease affecting chickens and causing serious economic loss. Although several studies have been conducted to characterize HVRs-S1 (Hyper-Variable Regions of Spike 1 gene) in Egypt, few of which aimed to characterize the major structural protein genes. In the present study, the genetic characterization of the major structural protein genes was carried out in 10 isolates selected from six governorates in 2019. Phylogenetically, the S1 gene was clustered into genotype GI-23 (variant II), with seven viruses that were clustered into Egy/Var II occurring in two subgroups (I, II) when aligned with previously isolated Egyptian strains. It had a specific character of 40 Amino Acids (AA) mutations except for IBV/EG/CV32/2019, which had 50 AA mutations, specifically in HVRs regions (HVRI, II, and III). The other three strains were clustered into Egy Var I with 17 AA mutations except IBV/EG/F859/ 2019, which had 15 AA mutations, compared to IBV/CU/4/2014 reference strain. The examined isolates had an additional glycosylation site at position 280 and one was missing at position 139 with the exception of two strains that only had an additional one, compared to IBV/CU/4/2014. The viruses in this study differed genetically from various vaccine seeds in the range of 69-83%. The Nucleocapsid, genetically characterized in the group of variant II (Egy/Var II) and the glycoprotein membrane genes genetically characterized in the variant group in a new sub-group with 11 and 9 AA mutations, respectively. The recombination event was only detected in the S1 gene in two isolates of IBV/EG/CV32/2019 and IBV/EG/F859/2019 from D274 and QX, respectively. In this regard, it is important to conduct continuous surveillance, pathogenicity study, and vaccine efficacy evaluation.
Keywords: Characterization, Infectious bronchitis virus, Major structure protein, Matrix, Nucleoprotein, Spike

[Full text-PDF] [XML] [Crossref Metadata]

Archive

Journal of World's Poultry Research

ISSN: 2322-455X

Frequency: Quarterly

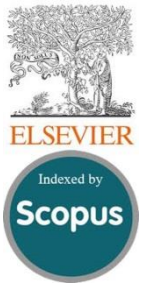
Current Issue: 2020, Vol: 10, Issue: 4 (December 25)

Publisher: SCIENCELINE

The Journal of World's Poultry Research (ISSN: 2322-455X) is an international, peer reviewed open access journal aims to publish the high quality material from poultry scientists' studies to improve domesticated birds production, food quality and safety ... view full aims and scope

www.jwpr.science-line.com

» Indexed/covered by SCOPUS, NLM Catalog (NLM ID: 101681042), DOAJ, HINARI, AGRIS, EBSCO, CIARDRING, NAAS (Score: 4.79), Ulrich's™/ ProQuest, PUBDB, ICV 2016= 91.33, TOCs, TIB, BASE, WorldCat, ISC-RICeST, EZB, WZB, Google Scholar...full index information



» Open access full-text articles is available beginning with Volume 1, Issue 1.

» Full texts and XML articles are available in ISC-RICeST, and AGRIS.

» This journal is in compliance with Budapest Open Access Initiative and International Committee of Medical Journal Editors' Recommendations.

ICMJE INTERNATIONAL COMMITTEE of MEDICAL JOURNAL EDITORS

BOAI 10

» High visibility of articles over the internet.

» This journal encourage the academic institutions in low-income countries to publish high quality scientific results, free of charges... view Review/Decisions/Processing/Policy

Editorial Offices:
Atatürk University, Erzurum 25100, Turkey
University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada
University of Maragheh, East Azerbaijan, Maragheh 55136, Iran
Homepage: www.science-line.com
Phone: +98 914 420 7713 (Iran); +90 538 770 8824 (Turkey); +1 204 8982464 (Canada)
Emails: administrator@science-line.com ; saeid.azar@atauni.edu.tr

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 545-555, December 25, 2020

Research Paper, PII: S2322455X2000062-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.62

Potential Biomarker for Fatty Liver Hemorrhagic Syndrome in Laying Hens

Lihui Zhu[1,2*], Rongrong Liao[1], Changfeng Xiao[1], Gensheng Zhu[2,3], Ning Wu[3], Yinyin Tu[2], and Changsuo Yang[1,2 **]

[1]*Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences, Shanghai, 201106, China*
[2]*National Poultry Research Center for Engineering and Technology, Shanghai, 201106, China*
[3]*Shanghai Haifeng Dafeng Poultry CO. LTD., Bright Food (Group) CO, LTD., Shanghai, 200085, China*

[*]Corresponding author's Email: zhulihui@saas.sh.cn; ORCID: 0000-0002-2931-6207
[**]Corresponding author's Email: yangchangsuo@189.cn; ORCID: 0000-0002-6285-3020

Received: 09 Oct. 2020
Accepted: 20 Dec. 2020

ABSTRACT

Fatty liver hemorrhagic syndrome is more common in laying hens with excess body weight (BW) and in the middle and late phase of egg production. However, no specific biomarkers in chickens can be used to diagnose liver steatosis or liver injury. The present study aimed to assess whether BW can be used to predict fatty liver in aged laying hens. This study also searched for potential plasma FLHS biomarkers. For these purposes, correlation among BW, relative weight of liver and abdominal fat, and plasma markers were analyzed in Hy-line brown laying hens. Furthermore, plasma levels of potential biomarkers were analyzed during the formation of fatty liver. Concentrations of triglycerides and total cholesterol were positively associated with BW in aged laying hens, while liver fat deposition was similar among chickens with different BW, indicating that BW cannot be used as the only criterion to discriminate aged laying hens with liver steatosis. A trend of increasing triglyceride, total cholesterol, fatty acid-binding protein 4 (FABP4), and lipoprotein lipase levels was found as age increased, and they were positively associated with BW indicating that they might be risk markers for FLHS in laying hens. The findings indicated that the plasma level of FABP4 was positively associated with the severity of fatty liver in aged laying hens. All the above results suggested that FABP4 might be a potential diagnostic indicator for FLHS.

Key words: Biomarker, Egg production, Fatty liver, Laying hens, Poultry

INTRODUCTION

Similar to nonalcoholic fatty liver disease (NAFLD), fatty liver hemorrhagic syndrome (FLHS) is related to liver degeneration resulted from excessive fat deposition in the liver causing liver rupture, hemorrhage, and sudden death of chickens (Wolford and Polin, 1974; Whitehead 1979). Excessive fat deposition is also observed in the abdominal cavity. This disease mainly occurs in commercial caged laying hens leading to reduced egg production and death, and accordingly significant economic losses (Julian, 2005). Nowadays, FLHS is the most common noninfectious cause of high mortality in laying hens (Trott et al., 2014). It is currently believed that nutrition, genetics, and environmental toxins are related to FLHS outbreaks in laying hen farms, and the low-protein and high-energy diet is the main reason for the occurrence of FLHS (Diaz et al., 1999; Choi et al., 2012; Rozenboim et al., 2016). There are no obvious symptoms in the early stage of the disease, and the diagnosis can only be confirmed by dissecting the chickens with sudden onset. Therefore, screening of specific biomarkers for liver steatosis or liver damage would help improve the quality and efficiency of large-scale farmed laying hens.

Conventionally, FLHS is more prevalent in birds with excess body weight (BW) and in the middle and late phases of egg production. However, the idea of whether BW can be used as a marker for FLHS has remained to be verified. Additionally, in birds, the liver is the major organ of lipid metabolism, and steady changes in liver lipid metabolism are the basis of various forms of fatty liver disease. Excess fatty acid supply or inhibited oxidation in the liver may result in enhanced synthesis of triglycerides (TG) and disordered very low-density lipoprotein (VLDL) synthesis and secretion, which are observed in laying hens with FLHS (Dong and Tong, 2019; Gao et al., 2019). Circulating alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are used as liver injury indicators and have been found to be increased in laying hens with

To cite this paper: Zhu L, Liao R, Xiao Ch, Zhu G, Wu N, Tu Y and Yang Ch (2020). Potential Biomarker for Fatty Liver Hemorrhagic Syndrome in Laying Hens. *J. World Poult. Res.*, 10 (4): 545-555. DOI: https://dx.doi.org/10.36380/jwpr.2020.62

FLHS (Hamid et al., 2019). Furthermore, many proteins in the blood, including those related to lipid metabolism, are synthesized in the liver, and the abundance and structure of these proteins also change in the development of liver disease, making them potential biomarkers for liver diseases including NAFLD (Kim et al., 2011; Vilar-Gomez and Chalasani, 2018). However, the clinical utility of these biomarkers has not been validated in chicken cohorts. The objectives of this study were, first, to assess whether BW can be used as an indicator for predicting fatty liver disease, and second, to screen potential plasma FLHS biomarkers in chickens.

MATERIALS AND METHODS

Ethical approval

All animal protocols used in this study were approved by the Ethics and Animal Welfare Committee of Shanghai Academy of Agricultural Sciences, China, and performed according to the guidelines established by 'the instructive notions with respect to caring for laboratory animals' issued by the Ministry of Science and Technology of the People's Republic of China (No. [2006] 398).

Animals and study design

A total number of 102,000 commercial Hy-line brown laying hens (20 to 65 weeks old) were raised in eight-high modular manure belt cages (Chore-Time Equipment Inc., Milford, IN, USA), with nine chickens per cage (dimensions of 60 × 65 × 45.5 cm) under standard commercial conditions. A standard commercial corn-soy diet (Shanghai Haifeng Dafeng Poultry Co. Ltd. Dafeng, Jiangsu, China) containing 16.00% crude protein, 0.73% lysine, 0.33% methionine, 4.1% calcium, 0.36% phosphorus, 0.17% salt, 19.3 ppm copper, 66 ppm iron, 80 ppm manganese, 0.3 ppm selenium, 2.2 ppm iodine, 80 ppm zinc, and 2700 kcal/kg calculated metabolizable energy was supplied for the layers. Feed and water were offered *ad libitum* throughout the experimental period. Separate groups of chickens were used for each experiment (Experiments 1-3).

Experiment 1

To verify whether BW can be used as a potential predictor of FLHS in aged laying hens, 60 laying hens aged 52 weeks were randomly divided into 4 groups according to the BW recommended by the Hy-line brown laying hens management guide (http://www.hy-line.co.uk/services/management-guides/). These four groups included low-grade weight (LW, 5% below standard weight), normal weight (NW, in standard weight range), overweight/obese (OW/Ob, <5% over standard weight), and seriously overweight/obese (SW/Ob, 5–10% over standard weight). The standard BW of Hy-line brown laying hens at 52 weeks should be 1.89 to 2.01 kg. To determine plasma concentrations of ALT, AST, TG, total cholesterol (TC), VLDL cholesterol (VLDL-C), fatty acid-binding protein 4 (FABP4), and lipoprotein lipase (LPL) blood samples from the experimental layers were obtained through the wing vein and collected in tubes containing EDTA as an anticoagulant. About 4 hours later, all the hens were killed by electric shock, and the abdominal fat and livers were removed and weighed immediately. The collected liver samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C for expression analysis of lipid metabolism-related genes. Another portion of the liver was fixed in 4% paraformaldehyde for histological analysis. The relative weight of abdominal fat and liver was calculated as the weight of abdominal fat or liver in 100 g body weight (g/100 g BW). The severity of fatty liver was evaluated by liver color and scored, as modified based on a study conducted by Choi et al. (2012). The fatty liver was scored on a scale from 1 to 4 as follows: **FS1**, normal liver with dark red; **FS2**, mild case of FLHS with mild yellow liver and hemorrhages; **FS3**, moderate case of FLHS with light yellowish red liver and hemorrhages; and **FS4**, large and massive hemorrhages with putty-colored livers. The standard for the fatty liver score according to Avinash (2007) is indicated in Figure 1.

Experiment 2

To assess mRNA expression of lipid metabolism-related genes, a total of 30 laying hens were killed by electric shock at specified ages (15 at the age of 27 weeks and 15 at the age of 52 weeks). All livers were harvested and checked for fatty liver by gross examination. The livers with dark red color and no hemorrhages were determined as normal liver and livers with light yellowish red color and hemorrhages were considered as fatty liver. From each group (normal and fatty liver), 4-5 samples were pooled together for mRNA expression analysis of some lipid metabolism-related genes, including apolipoprotein B (*APOB*), peroxisome proliferator-activated receptor α (*PPARα*), *PPARγ, LPL*, and *FABP4*.

Experiment 3

To determine the changes in concentrations of some biochemical indicators, including TG, TC, ALT, AST, FABP4, and LPL, with age, blood samples from 40 laying hens were collected every 5 weeks from age 27 to 52 weeks. At age of 52 weeks, all 40 laying hens were killed by electric shock, and checked for fatty liver as described above.

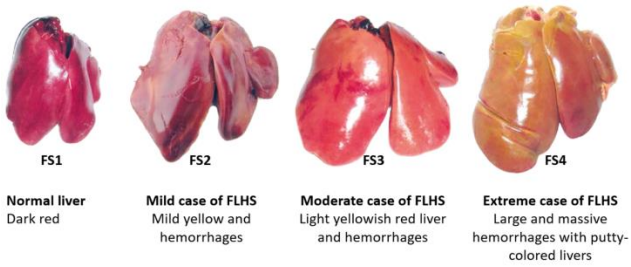


Figure 1. The standard for fatty liver score used for fatty liver examination in experiments 1-3. The color score, from 1 to 4 (from dark red to light yellowish-red), was judged by three investigators.

Histological analysis

The paraffin-embedded liver was cut into 5-μM sections and stained with hematoxylin and eosin. The degree of lipid accumulation was evaluated by the deposition of lipid droplets, which was quantified by ImageJ software (version 1.80, National Institutes of Health, Bethesda, MD, USA). The lipid accumulation was represented by the deposition of lipid droplets, with white pixel areas indicating fat vacuoles being divided by the total area scanned. The ImageJ software was used to quantify the staining of the liver section.

Biochemical analysis

Plasma samples were prepared by centrifugation (3,000 g for 15 min) and stored at –20 °C for future analysis. Concentrations of TG, TC, ALT, and AST were measured by colorimetric endpoint assays (A110-1-1, A111-1-1, C009-2-1, and C010-1-1 kits, respectively; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Total concentrations of plasma FABP4 and LPL were determined with chicken-specific commercial ELISA kits (Shanghai BlueGene Biotech Co. Ltd., China). Results were analyzed using the Thermo Multiskan Sky microplate reader (Thermo Fisher, Waltham, MA, USA). For liver TG and TC analysis, total proteins of liver were measured by bicinchoninic acid assay (kit A045-4-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the contents of liver TG and TC were measured by using kits A110-1-1 and A111-1-1, respectively (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All assays were performed according to the manufacturer's instruction.

Gene expression analysis

Chicken liver RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and checked on a Nanotrop Bioanalyzer (Agilent 2100; Palo Alto, CA, USA) for integrity analysis. The mRNA expression was detected by RT-qPCR in the liver. A PrimeScript RT reagent Kit (TaKaRa Biotechnology Co., Japan) was used to reverse transcribe RNA to cDNA, and qPCR was performed with SYBR Premix Ex Taq (TaKaRa Biotechnology Co., Japan) using an ABI Q5 Real-time PCR instrument (Applied Biosystems, Foster City, USA) at 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s, and 60 °C for 30 s. For RT-qPCR data analysis, each sample was run in triplicate. The mRNAs were normalized to *GAPDH* mRNA. The primer sequences are shown in Table 1. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analyses were carried out using SPSS version 21 (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way ANOVA and a *post hoc* Tukey test. P-value less than 0.05 was considered statistically significant. Spearman's correlation coefficient was used to test the relationship between various variables.

Table 1. Primers used in this study for RT-qPCR

Gene	Sequence (5'-3')
β-actin-F	AATGGCTCCGGTATGTGCAA
β-actin-R	GGCCCATACCAACCATCACA
GAPDH-F	GATGGGTGTCAACCATGAGAAA
GAPDH-R	CAATGCCAAAGTTGTCATGGA
PPARα-F	GCTTGTGAAGGTTGTAAGGGTT
PPARα-R	GACATTCCAACTGAAAGGCAC
PPARγ-F	GTCCTTCCCGCTGACCAAA
PPARγ-R	TCTCCTGCACTGCCTCCACA
FABP4-F	GGCCAAGCCTAATTTAACTATC
FABP4-R	TCCCATCCACCACTTTTCTC
APOB-F	ATACCCTGGGACTCTTGCCT
APOB-R	GAGAAGCTTTCAGGCTGGGT
LPL-F	ATTGCTGCCTCTTCTCCTTT
LPL-R	ATTGGTGACCTGCTTATGCTA

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PPARα: peroxisome proliferator-activated receptor α, PPARγ: peroxisome proliferator-activated receptor γ; FABP4: fatty acid-binding protein 4; APOB: apolipoprotein B; LPL: lipoprotein lipase.

RESULTS

Experiment 1

Liver and abdominal fat weight

In Experiment 1, different groups were significantly different in terms of BW ($P < 0.05$, Table 2). The amount of abdominal fat sequentially increased in chickens with

the increase of BW, although there was no significant difference between the normal BW and OW/Ob group ($P > 0.05$). The relative weight of abdominal fat was similar in both low and normal BW hens, whereas a significant increase of the relative weight of abdominal fat was observed in the overweight chickens (OW/Ob and SW/Ob groups), compared to the low BW group ($P < 0.05$). The relative weight of abdominal fat increased in the SW/Ob group, compared to the OW/Ob group ($P < 0.05$). There was no significant difference in the relative liver weight among chickens with different BW ($P > 0.05$). The concentration of TG was significantly higher in the SW/Ob hens, compared to the LW and the NW groups, while the obtained values of LW, NW, and OW/Ob hens were similar. The concentrations of TC were significantly lower in the LW laying hens than in the overweight hens (OW/Ob or SW/Ob), and the levels of AST were significantly increased in the NW, OW/Ob, and the SW/Ob groups in comparison to the LW hens. No significant differences in VLDL-C were observed among the four groups.

Correlation of body weight, relative weight of liver and abdominal fat with plasma markers

The abdominal fat pad weight, concentrations of TC and TG were significantly associated with BW ($P < 0.05$, Table 3). The concentrations of TC and TG were also positively correlated with the abdominal fat pad weight. The correlation between plasma TG and BW ($r = 0.662$) was stronger than that between plasma ALT and BW ($r = 0.108$), or plasma TC and BW ($r = 0.513$). Although other biomarkers were associated with each other (TG vs. ALT, $r = 0.224$; TG vs. TC, $r = 0.297$; VLDL-C vs. abdominal fad pad weight, $r = 0.169$), their correlations were insignificant. There was no significant correlation between liver weight and other biomarkers.

Table 2. Chicken body weights, relative weights of liver and abdominal fat, and plasma markers in the various body weight groups (Experiment 1)

Parameters	LW (n =15)	NW (n =15)	OW/Ob (n =15)	SW/Ob (n =15)
BW (g)	1665.71±40.03^{a}	1956.70±19.68^{b}	2084.43±14.17^{c}	2291.59±40.95^{d}
Relative weight, g/100 g BW				
Abdominal fat	2.49±0.21^{a}	3.05±0.27^{ab}	3.99±0.38^{b}	4.94±0.37^{c}
Liver	1.96±0.07	2.11±0.08	1.98±0.11	2.16±0.17
Plasma markers				
ALT(U/L)	17.03±2.68	11.36 ± 1.43	21.48±6.63	25.41±5.01
AST(U/L)	26.97±1.33^{a}	36.14±1.94^{b}	33.95±2.70^{b}	35.57±1.88^{b}
TC (mmol/L)	2.13±0.26^{a}	3.01±0.19^{ab}	4.13±0.50^{b}	3.57±0.27^{b}
VLDL-C (mmol/L)	2.25±0.21	3.64±0.50	2.95±0.34	3.34±0.76
TG (mmol/L)	13.45±1.10^{a}	16.58±1.20^{a}	23.17±2.09^{ab}	30.21±3.09^{b}

Values are expressed as mean ± SEM. ^{a,b,c} Mean values within a row with different superscript letters are significantly different ($P < 0.05$). ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, TG: Triglyceride, TC: Total cholesterol, VLDL-C: Very low-density lipoprotein cholesterol, FS: Fatty liver score, BW: Body weight, LW: Low grade weight, NW: Normal weight, OW/Ob: Overweight/obese, SW/Ob: Seriously overweight/obese

Table 3. Spearman correlation coefficients among the chicken body weights, relative weights of liver and abdominal fat, and serum markers (Experiment 1).

Variables	TG	TC	VLDL-C	AST	ALT	Relative weight, g/100 g BW	
						Abdominal fat weight	Liver weight
BW	0.662**	0.513**	0.235	0.248	0.108	0.658**	0.085
TG		0.297	0.097	0.152	0.224	0.318*	0.057
TC			-0.024	0.299	0.018	0.349**	0.075
VLDL-C				0.142	-0.093	0.169	-0.107
AST					-0.257	0.257	-0.003
ALT						-0.033	0.057
Relative abdominal fat weight, g/100 g BW							-0.187

BW: Body weight, TG: Triglyceride, TC: Total cholesterol, VLDL-C: Very low-density lipoprotein cholesterol, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase. * $P < 0.05$, ** $P < 0.01$.

Hepatic lipid accumulation

Pathological changes in the liver in all four groups at age 52 weeks are presented in Figure 2. The livers of overweight hens (OW/Ob and SW/Ob groups) were fragile and yellow, and some hemorrhagic spots appeared due to the high lipid accumulation when compared to layers with low BW (Figure 2a, b). The fatty liver score increased with BW. There was a significant increase in fatty liver score in the overweight groups (OW/Ob and SW/Ob groups), compared to the LW group ($P < 0.05$), while the fatty liver score in overweight hens (OW/Ob and SW/Ob groups) showed no significant difference between the two groups ($P > 0.05$, Figure 2c). However, the liver turned light yellow in the OW/Ob and SW/Ob groups indicating an increased fatty liver score, compared to the low and normal BW groups. To evaluate the consistency between liver lipid deposition and BW, the vacuolar areas of liver sections were quantified using image J software. The vacuolar area in the livers from overweight chickens (OW/Ob and SW/Ob groups) was significantly larger, compared to the low and normal BW groups ($P < 0.05$). No significant difference was observed between the two overweight groups (Figure 2d, $P > 0.05$). Concentrations of liver TG and TC were not significantly different in the chicken livers from the four groups ($P > 0.05$, Figure 2e).

Expression of lipid metabolism-related genes

The RT-qPCR analysis of lipid metabolism-related genes confirmed the changes in expression among the chickens with different levels of BW (Figure 3a). However, no regular expression of these genes was found among the layers with different BW values. As BW increased, *APOB* expression gradually decreased, and the mRNA expression of *APOB* decreased in the SW/Ob group compared to the LW group ($P < 0.05$), while it was not significantly different among the NW, OW/Ob, and SW/Ob groups ($P > 0.05$). Expression of *PPARα* was higher in the NW group, compared to the LW, OW/Ob, and SW/Ob groups ($P < 0.05$), however, no significant differences were observed among the three groups ($P > 0.05$). The mRNA expression of *FABP4* increased significantly in the NW, OW/Ob, and SW/Ob groups ($P < 0.05$), compared to the LW group, with the highest expression in the NW group. The expression of *PPARγ* was highest in the SW/Ob group, whereas there was no significant difference among the other three groups ($P > 0.05$). There was no significant difference in *LPL* mRNA expression among the four groups ($P > 0.05$) although it tended to increase with BW.

Plasma concentrations of FABP4 and LPL

Plasma concentrations of FABP4 and LPL in laying hens with different BW were also measured (Figure 3b). The concentration of FABP4 significantly increased as BW increased at age 52 weeks although no significant changes in LPL levels were observed among the four groups with different BW values. Plasma concentration of FABP4, measured by ELISA, was significantly upregulated and positively correlated with the fatty liver score (Figure 5c), indicating the diagnostic potential of plasma concentrations of FABP4. No significant correlation was found between plasma LPL and fatty liver score (data not shown).

Experiment 2

Increase of plasma FABP4 and LPL levels in chickens with fatty liver

To screen potential biomarkers in this chicken model of FLHS, five known lipid metabolism-related genes, including *APOB*, *PPARα*, *PPARγ*, *LPL*, and *FABP4* were selected for analyzing mRNA expression in normal and fatty livers. All samples from the laying hens of 52 weeks old were fatty liver. In laying hens of 27 weeks old, 12 out of 15 liver samples were normal; 3 liver with mild yellow color and hemorrhages were discarded from mRNA analysis. The results indicated that mRNA expression of *FABP4* and *LPL* significantly increased ($P < 0.05$), while the expression of *APOB*, *PPARα*, and *PPARγ* decreased ($P < 0.05$) in fatty livers, compared to normal livers (Figure 3c).

Experiment 3

Evaluation of changes in biochemical parameters with age of laying hens

The plasma concentrations of TG, TC, AST, ALT, FABP4, and LPL were measured in 40 laying hens every 5 weeks from age 27 to 52 weeks. The plasma concentrations of TG and TC displayed some variability among the individual layers. However, there was an increasing trend in TG levels with aging, compared to their concentrations in the laying hens at age 27 weeks (Figure 4a). At age 47 weeks, the concentrations of TG and TC were significantly higher than that at 32, 37, and 42 weeks. A significant decrease in the concentration of TC was observed in the plasma of the layers at age 52 weeks, compared to the obtained results at age 47 weeks, which was still higher than the obtained results of 27, 32, 37, and 42 weeks. No difference was found for the activities of ALT and AST among the six time points of

the layers although the activities of ALT and AST tended to increase with age. According to the recommended weight range, the experimental chickens were overweight since age 32 weeks (Figure 4b). From 27 to 52 weeks of age, plasma TG and TC concentrations were significantly correlated with BW ($r = 0.55$, $P < 0.01$ and $r = 0.61$, $P < 0.01$, respectively, Figure 4c). This trend in BW was consistent with the increasing trends of TG and TC concentrations. Plasma TG concentrations were positively associated with TC ($r = 0.68$, $P < 0.01$). The obtained results of ELISA revealed that FABP4 was significantly upregulated during 32-52 weeks of age (Figure 5a, $P < 0.05$). By age 52 weeks, the concentration of FABP4 and LPL was significantly higher than the age of 27 weeks ($P < 0.05$).

Correlation of FABP4 and LPL concentrations with other variables

Plasma FABP4 was positively correlated with LPL, TG, TC, and BW ($r = 0.29$, $r = 0.39$, $r = 0.20$, and $r = 0.31$, respectively, Figure 5b). Other markers were significantly associated with each other (LPL vs BW, $r = 0.18$; LPL vs TC, $r = 0.22$; and LPL vs TG, $r = 0.28$).

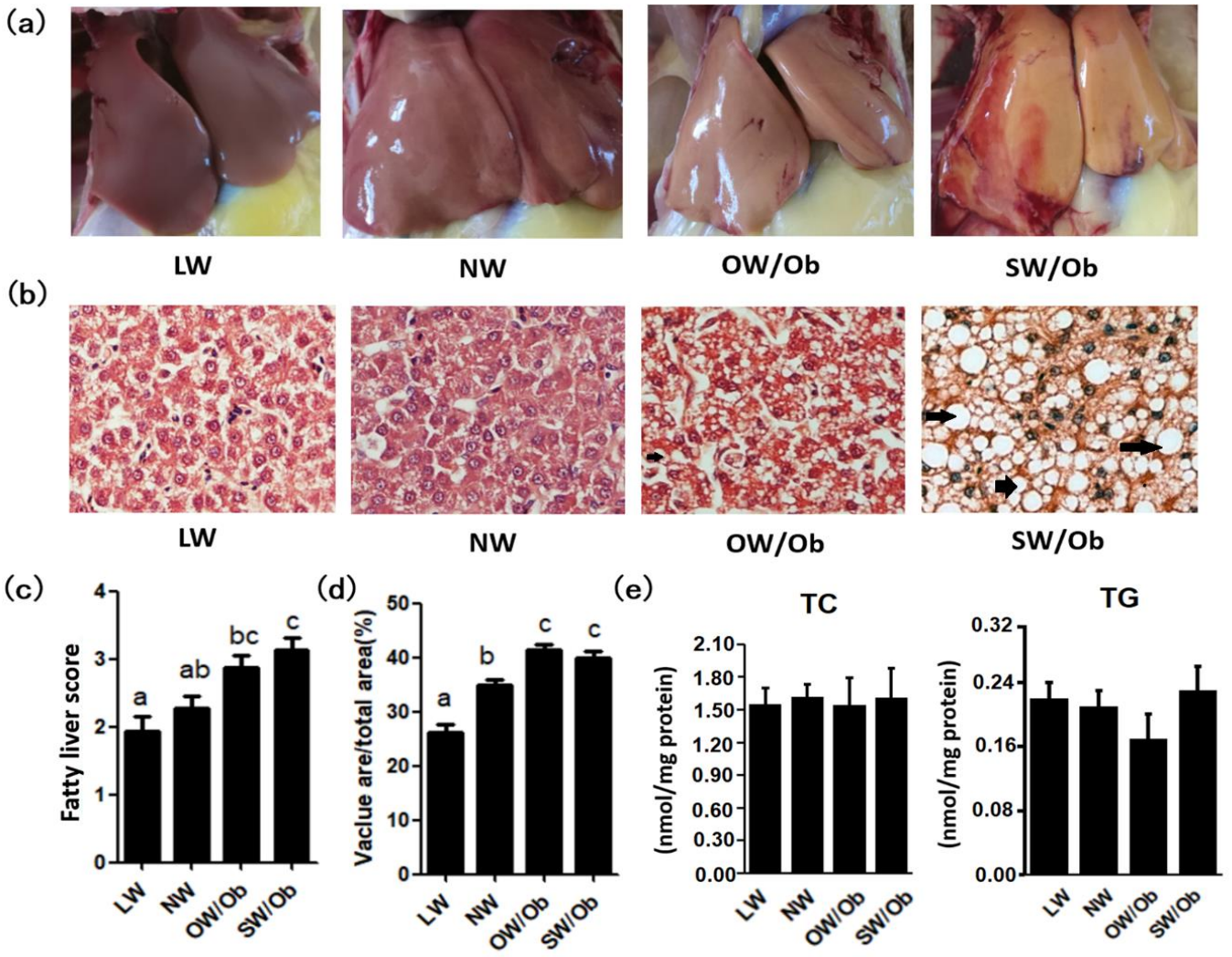


Figure 2. Results of evaluations of livers of chickens (52 weeks old) with different BWs. **a:** Representative images of various levels of liver hemorrhage. **b:** Representative images of histological sections of the liver after H&E staining. The arrow indicates the steatosis and lipid vacuolization inside the hepatocytes. Photographs were taken at 400× magnification. **c:** Mean fatty liver score in chickens with different BWs (score 1: normal liver with dark red; 2: mild case of FLHS with mild yellow liver and hemorrhages; 3: moderate case of FLHS, light yellowish red liver and hemorrhages; 4: severe case of FLHS, large and massive hemorrhages with putty-colored livers). **d:** Quantified results of fat vacuoles area within the liver section. **e:** Liver TG and TC concentrations in chickens with different BW. LW: Low body weight, NW: Normal body weight, OW/Ob: Overweight/obese, SW/Ob: Seriously overweight/obese, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, TG: Triglyceride, TC: Total cholesterol.

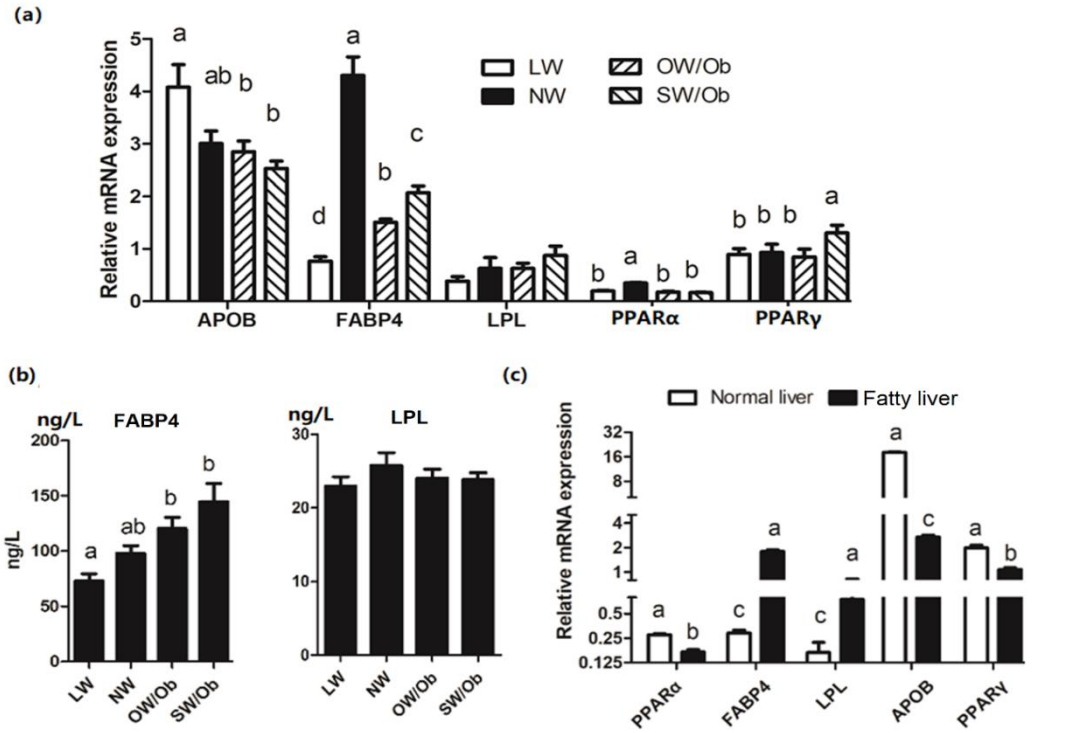


Figure 3. Evaluation of lipid metabolism-related parameters in relation to BW and liver condition. **a:** RT-qPCR analysis of lipid metabolism-related genes in livers of laying hens with different BW. **b:** Chicken plasma concentrations of FABP4 and LPL in laying hens with different BW (n = 15/BW group). **c:** RT-qPCR analysis of lipid metabolism-related genes in normal and fatty livers collected from laying hens of 27 and 52 weeks old, respectively. FABP4: Fatty acid-binding protein 4, LPL: Lipoprotein lipase, BW: Body weight, LW: Low body weight, NW: Normal body weight, OW/Ob: Overweight/obese, SW/Ob: Seriously overweight/obese.

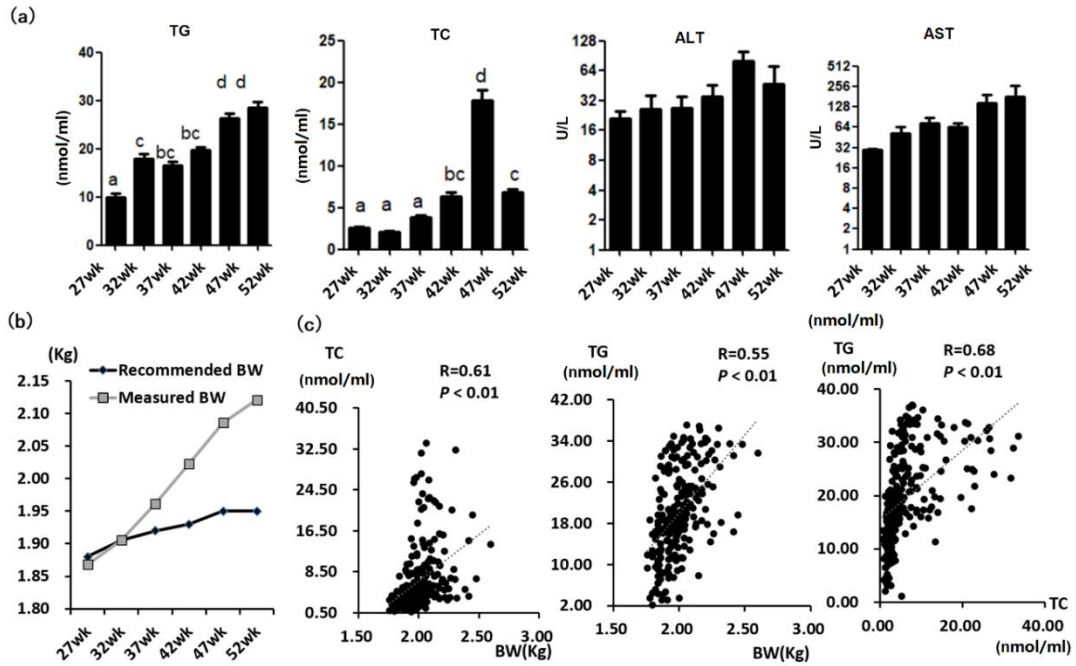


Figure 4. Evaluation of changes in biochemical parameters with age of laying hens. **a:** Plasma concentrations of TG, TC, ALT, and AST at six time points in laying hens. **b:** Comparison of measured BW of laying hens at different ages with recommended BW. **c:** Correlation of BW with TG and TC; Correlation of TG with TC. Values are expressed as mean ± SEM. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, TG: Triglyceride, TC: Total cholesterol, FLHS: Fatty liver hemorrhagic syndrome, BW: Body weight.

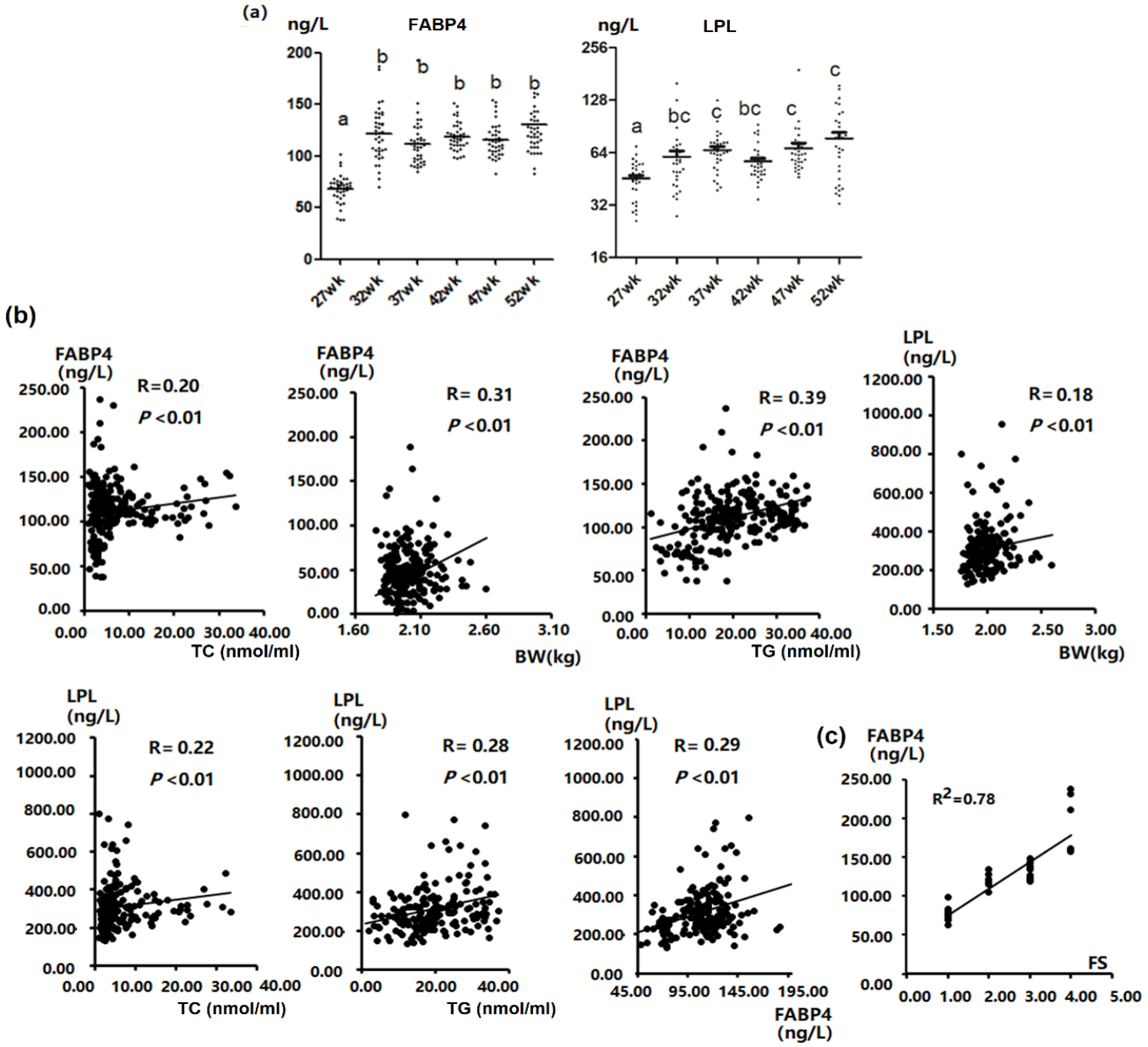


Figure 5. Evaluation of changes in FABP4 and LPL levels with age of laying hens. **a:** Scatter dot plot showing plasma concentrations of FABP4 and LPL in laying hens (n = 40) at different ages. **b:** Spearman's correlation analyses between FABP4 and LPL concentrations and other variables using data measured from 27 to 52 weeks of age. **c:** Correlation between plasma concentrations of FABP4 and FS of chickens (FS1: normal liver, n = 13; FS2: mild FLHS, n = 12; FS3: moderate FLHS, n =15; FS4: severe FLHS, n =10). FS: Fatty liver score, FABP4: Fatty acid-binding protein 4, LPL: Lipoprotein lipase, BW: Body weight.

DISCUSSION

FLHS is a metabolic disease characterized by excessive fat accumulation in the liver, accompanied by a hemorrhagic and pale liver, causing sudden death of laying hens (Shini et al., 2012). Signs of this metabolic disease are difficult to identify when the birds are alive, and it can only be determined through dissection. Therefore, it is urgent to find reliable noninvasive biomarkers to diagnose FLHS and monitor laying hens at an earlier time point when diet changes and potential health treatments can be used purposefully. The present study examined the relationship between BW and serum lipoproteins, and identified FABP4 as a potential novel circulating biomarker of FLHS.

Based on findings addressing production, aged layers have an increased risk of FLHS, and the BW of these layers is always higher than recommended values in the breeding manual (Dong and Tong, 2019). Previous studies have indicated that the occurrence of FLHS is the result of abnormal accumulation of liver fat, mainly due to the large increase in liver TG, and the abnormal accumulation of fat caused by dysfunction in liver fat metabolism (Dong and Tong, 2019; Shini et al., 2019; Zhuang et al., 2019; Shini et al., 2020). The present study demonstrated that concentrations of TG, TC, and VLDL-C were positively correlated with BW in aged laying hens, and the amount of abdominal fat and the concentration of TG in aged layers significantly increased with BW gain. Lipid accumulation and hemorrhage were more severe in the overweight layers of the low BW group. These results suggested that the higher the weight, the higher the risk of FLHS in aged layers. However, it is worth noting that no difference was observed in the relative liver weight and the concentration of VLDL-C among chickens with different values of BW. The concentrations of TG were also similar among the NW and OW/Ob group hens. Also, the relative weight of abdominal fat was similar between LW and NW hens. In addition, no significant difference in the fatty liver score was observed between layers in adjacent BW groups. Based on this, it can be found that the differences between layers in adjacent BW groups were relatively small, and BW within the standard range recommended by the manufacturer could not be used as an indicator of FLHS in aged layers. Thus, these findings indicated that BW of laying hens should be monitored at all times during production, but BW cannot be used as the sole criterion to discriminate aged laying hens with liver steatosis.

TG represent the main form of storage and transport of fatty acids in cells and circulating system, and they have been suggested to have the potential for diagnosis of NAFLD (Safaei et al., 2016). Evidence also suggests that TC is a risk factor for NAFLD, and as the level of TC improves, the incidence of NAFLD increases as well (Ballestri et al., 2016; Ren et al., 2019). High levels of TG and TC were also reported in previous studies in laying hens with FLHS (Dong and Tong, 2019; Gao et al., 2019). To find potential serum markers for early prediction of FLHS, changes in the plasma concentrations of TG and TC were examined every 5 weeks from 27 weeks (normal liver) to 52 weeks (FLHS was confirmed through dissection at the end of the experiment) of age in 40 laying hens. Furthermore, the serum activities of AST and ALT were examined during this period as liver injury indicators. It was found that TG and TC levels, which were positively associated with BW, increased as chickens grew older. This means that TG and TC are good risk markers for FLHS although further confirmation is needed since the current study did not address the correlation of FLHS morphology with TG and TC levels at different periods. Furthermore, no difference was found in the activities of ALT and AST among the six time points, indicating that the classic liver injury indicators, AST and ALT, were unable to predict chicken FLHS, which was consistent with the conclusion drawn for NAFLD (Ekstedt et al., 2006). The results were also in line with a previous study conducted by Diaz et al. (1999) who found no clear correlation between plasma enzyme activities (AST, ALT, and lactate dehydrogenase) and the degree of liver hemorrhage in laying hens.

Finally, five known lipid metabolism-related genes, *APOB*, *PPARα*, *PPARγ*, *LPL*, and *FABP4*, were selected for mRNA expression analysis in fatty and normal livers. Expression of *APOB*, *PPARα*, and *PPARγ* decreased in fatty livers, compared to the normal liver as opposed to the results reported by Li et al. (2015). They analyzed the liver transcriptome of young chickens (age 20 weeks) and laying hens (age 30 weeks) by sequencing, and found that the mRNA levels of fatty acid uptake and transport (*PPARγ*, *APOB*, and *FABP3*) increased in the liver of laying hens (Li et al., 2015). This difference may be due to the age of the birds studied. The current study examined the expression of lipid metabolism-related genes in the liver of aged chickens with FLHS, while Li et al. (2015) focused on younger birds at peak production. A similar result was reported by Song et al. (2017), in which obvious decreases in apolipoprotein (apo) A-I and apoB100 levels were observed in the hepatic tissues. The authors reported that expression of apoA-I decreased on day 60 while the apoB100 levels slightly increased on day 30 and then decreased on day 60 in the liver of an FLHS model induced by a low-protein high-energy diet, and they speculated that this decrease was due to liver injury from FLHS (Song et al., 2017). APOB plays an important role in the hepatic export of triacylglycerols by interacting with TG, cholesterol, and phospholipids (Kessler et al., 2014; Devaraj and Jialal, 2020). The fatty acid oxidation related gene *PPARα*, together with *PPARγ* and *PPARβ/δ*, is involved in many aspects of lipid metabolism (Bougarne et al., 2018). Hepatocyte PPARα deletion impairs fatty acid catabolism resulting in hepatic lipid accumulation (Gao et al., 2015; Montagner et al., 2016). Hence, the downregulation of *APOB* and *PPARα* may contribute to the hepatic lipid accumulation in the liver of aged laying hens with FLHS as suggested by Lu et al. (2019).

The mRNA expression of *FABP4* and *LPL* significantly increased in the liver of FLHS chickens. FABP4 is an important lipid chaperone that plays an important role in lipid-mediated cellular physiological processes and metabolism. FABP4 is mainly expressed in adipocytes and macrophages, and high expression of FABP4 is closely related to the metabolic syndrome caused by obesity (Kralisch and Fasshauer, 2013). Elevated expression of hepatic FABP4 is positively correlated with the severity of NAFLD (Thompson et al., 2018). High levels of serum FABP4 are also reported in NAFLD patients, however, its use as a prognostic marker in serum is still controversial (Koh et al., 2009; Kim et al., 2011; Coilly et al., 2019). LPL transports plasma lipids into tissues and plays an important role in the regulation of lipid metabolism and energy balance (Li et al., 2014). In contrast, there is almost no LPL expression in normal adult liver, while increased hepatic *LPL* mRNA expression is observed in obese humans, which is postulated to contribute to hepatic TG accumulation (Pardina et al., 2009). Consistent with the obtained results of NAFLD, mRNA analysis indicated that expression of *FABP4* and *LPL* was upregulated in the liver of laying hens with FLHS. Plasma FABP4 and LPL concentrations sequentially increased in chickens with increased BW indicating that plasma FABP4 and LPL might be risk markers for FLHS in laying hens. Accordingly, there was a positive correlation between the plasma level of the FABP4 and the severity of fatty liver in aged laying hens suggesting that FABP4 might be a potential diagnostic marker for FLHS.

CONCLUSION

In conclusion, plasma TC, TG, FABP4, and LPL are positively associated with BW, and risk of FLHS. Determination of these biomarkers in plasma samples from FLHS hens could provide a rapid and first-line diagnosis of FLHS for clinical application.

DECLARATIONS

Acknowledgments

This work was supported by the SAAS Program for Excellent Research Team (SPERT) under Grant (B-03) and China Agriculture Research System under Grant (CARS-40-K03).

Competing interests

The authors declare that they have no competing interests.

Author contributions

Professor CY supported the funding. Dr RL and CX did the RT-qPCR and ELISA assay. Mr NW, Mr GZ, and Ms YT collected the samples. Associate professor LZ designed the experiments, analyzed the data, did the experiments, and wrote the manuscript. All authors read and approved the final manuscript.

REFERENCES

Avinash Dhawale (2007). The liver: A big organ with a big role. World poultry, 23(10): 34-36. Available at: https://www.poultryworld.net/Breeders/Nutrition/2007/10/The-liver-A-big-organ-with-a-big-role-WP006905W/

Ballestri S, Nascimbeni F, Romagnoli D, and Lonardo A (2016). The independent predictors of non-alcoholic steatohepatitis and its individual histological features. Insulin resistance, serum uric acid, metabolic syndrome, alanine aminotransferase and serum total cholesterol are a clue to pathogenesis and candidate targets for treatment. Hepatology Research, 46(11): 1074-1087. DOI: http://www.doi.org/10.1111/hepr.12656

Bougarne NWB, Desmet SJ, Deckers J, Ray DW, Staels B, and De Bosscher K (2018). Molecular actions of PPARα in lipid metabolism and inflammation. Endocrine Reviews, 39(5): 760-802. DOI: http://www.doi.org/10.1210/er.2018-00064

Choi YI, Ahn HJ, Lee BK, Oh ST, An BK, and Kang CW (2012). Nutritional and hormonal induction of fatty liver syndrome and effects of dietary lipotropic factors in egg-type male chicks. Asian-Australasian Journal of Animal Sciences, 25(8): 1145-1152. DOI: http://www.doi.org/10.5713/ajas.2011.11418

Coilly A, Desterke C, Guettier C, Samuel D, and Chiappini F (2019). FABP4 and MMP9 levels identified as predictive factors for poor prognosis in patients with nonalcoholic fatty liver using data mining approaches and gene expression analysis. Scientific Reports, 9(1): 19785. DOI: http://www.doi.org/10.1038/s41598-019-56235-y

Devaraj S, and Jialal I (2020). Biochemistry, Apolipoprotein B. In StatPearls. StatPearls Publishing. Available at: https://www.ncbi.nlm.nih.gov/books/NBK538139/

Diaz GJ, Squires EJ, and Julian RJ (1999). The use of selected plasma enzyme activities for the diagnosis of fatty liver-hemorrhagic syndrome in laying hens. Avian Diseases, 43: 768-773. Available at: https://pubmed.ncbi.nlm.nih.gov/10611993/

Dong X, and Tong J (2019). Different susceptibility to fatty liver-haemorrhagic syndrome in young and older layers and the interaction on blood LDL-C levels between oestradiols and high energy-low protein diets. British Poultry Science, 60(3): 265-271. DOI: http://www.doi.org/10.1080/00071668.2019.1571164

Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, and Kechagias S (2006). Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology, 44(4): 865-873. DOI: http://www.doi.org/10.1002/hep.21327

Gao Q JY, Yang G, Zhang X, Boddu PC, Petersen B, Narsingam S, Zhu YJ, Thimmapaya B, Kanwar YS, and Reddy JK (2015). PPARα-deficient ob/ob obese mice become more obese and manifest severe hepatic steatosis due to decreased fatty acid oxidation. American Journal of Pathology, 185(5): 1396-1408. DOI: http://www.doi.org/10.1016/j.ajpath.2015.01.018

Gao X LP, Wu C, Wang T, Liu G, Cao H, Zhang C, Hu G, and Guo X (2019). Effects of fatty liver hemorrhagic syndrome on the AMP-activated protein kinase signaling pathway in laying hens. Poultry Science, 98(5): 2201-2210. DOI: http://www.doi.org/10.3382/ps/pey586

Hamid H ZJ, Li WX, Liu C, Li ML, Zhao LH, Ji C, and Ma QG (2019). Interactions between the cecal microbiota and non-alcoholic

steatohepatitis using laying hens as the model. Poultry Science, 98(6): 2509-2521. DOI: http://www.doi.org/10.3382/ps/pey596

Julian RJ (2005). Production and growth related disorders and other metabolic diseases of poultry--a review. Veterinary Journal, 169(3): 350-369. DOI: http://www.doi.org/10.1016/j.tvjl.2004.04.015

Kessler EC, Gross JJ, Bruckmaier RM, and Albrecht C (2014). Cholesterol metabolism, transport, and hepatic regulation in dairy cows during transition and early lactation. Journal of Dairy Science, 97(9): 5481-5490. DOI: http://www.doi.org/10.3168/jds.2014-7926

Kim YC, Cho YK, Lee WY, Kim HJ, Park JH, Park DI, Sohn CI, Jeon WK, Kim BI et al. (2011). Serum adipocyte-specific fatty acid-binding protein is associated with nonalcoholic fatty liver disease in apparently healthy subjects. Journal of Nutritional Biochemistry, 22(3): 289-292. DOI: http://www.doi.org/10.1016/j.jnutbio.2010.02.007

Koh JH, Shin YG, Nam SM, Lee MY, Chung CH and Shin JY (2009). Serum adipocyte fatty acid-binding protein levels are associated with nonalcoholic fatty liver disease in type 2 diabetic patients. Diabetes Care, 32(1): 147-152. DOI: http://www.doi.org/10.2337/dc08-1379

Kralisch S, and Fasshauer M (2013). Adipocyte fatty acid binding protein: a novel adipokine involved in the pathogenesis of metabolic and vascular disease? Diabetologia, 56(1): 10-21. DOI: http://www.doi.org/10.1007/s00125-012-2737-4

Li H WT, Xu C, Wang D, Ren J, Li Y, Tian Y, Wang Y, Jiao Y, Kang X, and Liu X (2015). Transcriptome profile of liver at different physiological stages reveals potential mode for lipid metabolism in laying hens. BMC Genomics, 16: 763. DOI: http://www.doi.org/10.1186/s12864-015-1943-0

Li Y, He PP, Zhang DW, Zheng XL, Cayabyab FS, Yin WD, and Tang CK (2014). Lipoprotein lipase: from gene to atherosclerosis. Atherosclerosis, 237(2): 597-608. DOI: http://www.doi.org/10.1016/j.atherosclerosis.2014.10.016

Livak KJ, and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. Methods, 25(4): 402-408. DOI: http://www.doi.org/10.1006/meth.2001.1262

Lu Z, He XF, Ma BB, Zhang L, Li JL, Jiang Y, Zhou GH, and Gao F (2019). Increased fat synthesis and limited apolipoprotein B cause lipid accumulation in the liver of broiler chickens exposed to chronic heat stress. Poultry Science,98(9): 3695-3704. DOI: http://www.doi.org/10.3382/ps/pez056

Montagner A, Polizzi A, Fouché E, Ducheix S, Lippi Y, Lasserre F, Barquissau V, Régnier M, Lukowicz C et al. (2016). Liver PPARα is crucial for whole-body fatty acid homeostasis and is protective against NAFLD. Gut,65(7): 1202-1214. DOI: http://www.doi.org/10.1136/gutjnl-2015-310798

Pardina E, Baena-Fustegueras JA, Llamas R, Catalán R, Galard R, Lecube A, Fort JM, Llobera M, Allende H, Vargas V et al. (2009). Lipoprotein lipase expression in livers of morbidly obese patients could be responsible for liver steatosis. Obesity Surgery, 19(5): 608-616. DOI: http://www.doi.org/10.1007/s11695-009-9827-5

Ren XY, Shi D, Ding J, Cheng ZY, Li HY, Li JS, Pu HQ, Yang AM, He CL, Zhang JP et al. (2019). Total cholesterol to high-density lipoprotein cholesterol ratio is a significant predictor of nonalcoholic fatty liver: Jinchang cohort study. Lipids in Health and Disease, 18(1): 47. DOI: http://www.doi.org/10.1186/s12944-019-0984-9

Rozenboim I, Mahato J, Cohen NA, and Tirosh O (2016). Low protein and high-energy diet: a possible natural cause of fatty liver hemorrhagic syndrome in caged White Leghorn laying hens. Poultry Science, 95(3): 612-621. DOI: http://www.doi.org/10.3382/ps/pev367

Safaei A, Arefi Oskouie A, Mohebbi SR, Rezaei-Tavirani M, Mahboubi M, Peyvandi M, Okhovatian F, and Zamanian-Azodi M (2016). Metabolomic analysis of human cirrhosis, hepatocellular carcinoma, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis diseases. Gastroenterology and Hepatology from Bed to Bench, 9(3), 158-173. Available at: https://pubmed.ncbi.nlm.nih.gov/27458508/

Shini A, Shini S, Filippich LJ, Anderson ST, and Bryden WL (2012). Role of inflammation in the pathogenesis of fatty liver haemorrhagic syndrome in laying hens. Proceedings of the Australian Poultry Science Symposium. University of Sydney Poultry Research Foundation. Available at: https://espace.library.uq.edu.au/view/UQ:272199

Shini S, Shini A, and Bryden WL (2019). Unravelling fatty liver haemorrhagic syndrome: 2. Inflammation and pathophysiology. Avian Pathology, 49(2): 131-143. DOI: http://www.doi.org/10.1080/03079457.2019.1682119

Shini S, Shini A, and Bryden WL (2020). Unravelling fatty liver haemorrhagic syndrome: 1. Oestrogen and inflammation. Avian Pathology,49(1): 87-98. DOI: http://www.doi.org/10.1080/03079457.2019.1674444

Song Y, Ruan J, Luo J, Wang T, Yang F, Cao H, Huang J, and Hu G (2017). Abnormal histopathology, fat percent and hepatic apolipoprotein A I and apolipoprotein B100 mRNA expression in fatty liver hemorrhagic syndrome and their improvement by soybean lecithin. Poultry Science, 96(10): 3559-3563. DOI: http://www.doi.org/10.3382/ps/pex163

Thompson KJ, Austin RG, Nazari SS, Gersin KS, Iannitti DA, and McKillop IH (2018). Altered fatty acid-binding protein 4 (FABP4) expression and function in human and animal models of hepatocellular carcinoma. Liver International : Official Journal of the International Association for the Study of the Liver, 38(6): 1074-1083. DOI: http://www.doi.org/10.1111/liv.13639

Trott KA, Giannitti F, Rimoldi G, Hill A, Woods L, Barr B, Anderson M, and Mete A (2014). Fatty liver hemorrhagic syndrome in the backyard chicken: a retrospective histopathologic case series. Veterinary Pathology, 51(4): 787-795. DOI: http://www.doi.org/10.1177/0300985813503569

Vilar-Gomez E, and Chalasani N (2018). Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. Journal of Hepatology, 68(2): 305-315. DOI: http://www.doi.org/10.1016/j.jhep.2017.11.013

Whitehead CC (1979). Nutritional and metabolic aspects of fatty liver disease in poultry. The Veterinary Quarterly, 1(3): 150-157. DOI: http://www.doi.org/10.1080/01652176.1979.9693738

Wolford JH, and Polin D (1974). Induced fatty liver-hemorrhagic syndrome (FLHS) and accumulation of hepatic lipid in force-fed laying chickens. Poultry Science, 53(1): 65-74. DOI: http://www.doi.org/10.3382/ps.0530065

Zhuang Y, Xing C, Cao H, Zhang C, Luo J, Guo X, and Hu G (2019). Insulin resistance and metabonomics analysis of fatty liver haemorrhagic syndrome in laying hens induced by a high-energy low-protein diet. Scientific Reports, 9(1): 10141. DOI: http://www.doi.org/10.1038/s41598-019-46183-y

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 556-564, December 25, 2020

Research Paper, PII: S2322455X2000063-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.63

Immune-Complex Infectious Bursal Disease Virus versus Live Attenuated Vaccines to Protect SPF Chicken against Very Virulent Virus Challenge

Moustafa S. Abou El-Fetouh, Magdy H. Hafez, El-Sayed R. El-Attar, and Mohammed Ezzat El-Agamy*

Department of Pathology, Faculty of Veterinary Medicine, Zagazig University, Egypt
*Corresponding author's Email: aboelagamy2005@yahoo.co.uk; ORCID: 0000-0003-2416-2947

Received: 24 Sept. 2020
Accepted: 05 Nov. 2020

ABSTRACT

In this study infectious bursal disease (IBD) vaccinations were evaluated against very virulent IBD (vvIBDV) challenge and were compared. A total of 120-day-old white Leghorn SPF chickens were divided into 6 groups (each was 20 birds). Two groups were vaccinated on either day 1 with an immune-complex vaccine. The second groups were vaccinated at days 9 and 14 of age using intermediate and intermediate plus IBD vaccines, respectively the balance groups are controls. All vaccines were administered according to the manufacturer's instructions. The challenge was conducted on the 16 days of age using 10^5 EID_{50} /0.1 ml of a vvIBDV strain via the oculonasal route. The antibody immune response was monitored in all groups at 14, 21, 28, and 35 days of age. The performance, bursal gross lesions, challenge virus detection, and bursal histopathology were evaluated in vaccinated non challenged and vaccinated challenged birds at days 21 and 28 of age. All vaccinated groups were protected against the vvIBDV challenge compared to 40% mortality in the challenge control group. Both the immune-complex and live attenuated IBD vaccine groups showed similar bursa body weight (BB) ratios compared to the negative control group. The immune-complex vaccinated groups antibody titers were significantly higher except on 28th day of age. Upon challenge, the intermediate/intermediate plus vaccinated challenged group showed higher antibody titers at 21 and 35th with the challenge virus detection and quantification on day 28. The immune-complex vaccinated challenged group developed milder bursal histopathology signs but no differences between the 2 vaccine programs were seen. It can be understandable, the use of either immune-complex vaccine at day-old or early vaccination with intermediate followed by intermediate plus live attenuated IBD vaccines induced protective antibody titers and protect chickens against an early vvIBDV challenge. The suggested schedules need further evaluation in commercial broilers with maternal derived IBD antibodies to simulate field conditions.

Keywords: Immune-Complex vccine, Infectious Bursal Disease, Live Attenuated Vaccine, SPF Chicken

INTRODUCTION

Infectious Bursal Disease (IBD) is an acute and highly contagious disease in chickens at 3 weeks of age and older causing high mortality and immunosuppression leading to a variety of secondary infections and a decreased response to vaccinations (Eterradossi and Saif, 2020). IBDV belongs to the *Birnaviridae* family within the genus Avibirnavirus (Delmas et al., 2019). The virus is non-enveloped, single shelled with a diameter of 60 to 70 nm (Alkie and Rautenschlein, 2016).

IBDV has a predilection for the immature actively dividing B lymphocytes and causes lytic infection of IgM bearing B cells resulting in the decrease in circulating IgM^+ cells (Sivanandan and Maheswaran, 1980; Dey et al., 2019). Infected chicken produces less level of antibodies against the antigen (Ingraoet al., 2013). IBDV induced humoral deficiency is reversible and overlaps with the restoring of bursal morphology (Sharmaet al., 2000).

Three main clinical symptoms of the disease were reported and include the classical form caused by the classic moderate virulent strains of IBDV and manifested by acute depression followed by typical signs and lesions with 10–50% mortality. The acute form is caused by very virulent strains of IBDV (vvIBDV) characterized by acute progressive and typical clinical signs resulting in high mortality rates on affected farms (50–100%) (Stoute et al., 2009; Ewies et al., 2017; Eterradossi and Saif, 2020). Finally, the immunosuppressive form, principally described in the United States, is caused by low-

pathogenicity strains of IBDV and variant strains (e.g. Delaware strains) with few clinical signs, no mortality but with marked bursal lesions and concurrent infections with other agents (Dey et al., 2019; Shehata et al., 2019).

The available live vaccines against IBDV are categorized as "mild", "intermediate" and "hot" according to their degree of virulence (Rautenschleinet al., 2003). Killed virus vaccines in oil-adjuvant can prolong the duration of immunity in breeder flocks in addition to containing both standard and variant IBDV strains. Therefore, maternal antibody profiling of the breeder flock should be done to assess the effectiveness of vaccination and the persistence of antibody (Eterradossi and Saif, 2020). Mild vaccines are not very effective in the presence of high levels of maternal antibodies or against very virulent strains of IBDV. Intermediate and hot vaccines are much more effective but may induce moderate to severe lesions in the bursa of Fabricius (BF) (Camilotti et al., 2016).

To overcome the problem of maternal immunity interference, the IBDV vectored vaccines were developed. For instance, herpesviruses expressing the surface viral protein 2 (VP2) of IBDV (Perozo et al., 2009) and immune complex vaccines containing an IBDV-specific antibody and live-attenuated IBDV (Schat et al., 2011). Both types are commercially available and administered in-ovo or at one-day of age without maternal immunity interference (Muller et al., 2012).

In this study, the administration of immune-complex IBD vaccine (Bursaplex®) at day-old was compared with the use of an intermediate followed by an intermediate plus vaccines at 9 and 14 day-old, respectively, in SPF chickens. The comparison items included the clinical protection, bursal pathology, serology as well as detection and quantification of the challenge virus.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were reviewed and approved by the Faculty of Veterinary Medicine, Zagazig University, Egypt (FVM/ZAG-191207).

Vaccines and viruses

Bursaplex® (Zoetis, USA) is an immune-complex IBD vaccine that contains an embryo origin IBD live strain in conjunction with bursal disease antiserum and recommended for subcutaneous injection of chickens at one day of age. Bursine®-2 "intermediate live attenuated IBD vaccine" and Bursine plus® "intermediate Plus live attenuated IBD vaccine" (Zoetis, Belgium) were used. The challenge vvIBDV strain BSU-03-2016 (acc.no. KX077978) was retrieved from the repository of the Poultry Diseases Department, Faculty of Veterinary Medicine, Beni-Suef University, Egypt. The virus was propagated and titrated in 11 day-old SPF embryonated chicken eggs (Eterradossi and Saif, 2016).

Experimental design

A total of 120-day-old white Leghorn SPF chickens were divided into 6 groups (20 birds) placed in a negative pressure chicken isolator. Birds were vaccinated on either day 1 or days 9and 14 of age according to the experimental design summarized in table 1. All vaccines were administered according to the manufacturer's instructions. The challenge was conducted on the 16th day of age using 10^5 EID_{50} /0.1 ml of the vvIBDV strain via the oculonasal route (Eterradossi and Saif, 2016).

IBDV ELISA testing

Collected chicken sera were checked for IBD specific antibodies using ID Screen® IBD Indirect ELISA kit (IDvet, France) according to manufacturer instructions.

IBDV RT-PCR detection

Vaccinal and vvIBDV detection and quantification in 5 pooled bursal homogenates collected at the 28th day of age were conducted using qualitative Kylt® IBDV Pathotyping kits (AniCon Labor GmbH, Germany). The RNA was extracted using Kylt® RNA/DNA Purification kit (AniCon Labor GmbH, Germany) according to the manufacturer's instructions. The reaction performed in a 20 µl volume consisted of 10 µl of 2x RT-qPCR-Mix, 6 µl of Detection-Mix, and 4.0 µl of the tested RNA samples. The Kylt® kit thermal Profile III was; reverse transcription at 50 °C for 10 min, activation of Polymerase at 95 °C for 1 min, and 42 cycles of Denaturation at 95 °C for 10 sec, annealing and extension at 55°C for 1 min. The fluorescence detection channels were fluorescein (FAM) for vvIBDV, Cy5 for vaccinal IBDV, and Hexachloro-Fluorescein (HEX) for the internal kit control.

Histopathology

The bursa of Fabricius, thymus, spleen, and kidneys were collected from 5 chickens at 21 and 28th days of age. The formalin-fixed paraffin-embedded specimens were sectioned (4–5 µm) and stained with hematoxylin and

eosin stain and examined microscopically (Suvarna et al., 2018).

Statistical analysis

The differences in body weights, bursal body weight ratios (bursal weight/bodyweight×1000), and ELISA antibody titers were estimated using one-way ANOVA with multiple comparison Tukey's post-test through GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, *www.graphpad.com*).

Table 1. Experimental design of different vaccination schedules, sampling, and sample testing

Groups	Vaccination	Challenge at 16th day[1]	Monitoring, sampling, and measurements
Control negative	-	-	- ELISA: IBD ELISA at 0 day - Daily monitoring for clinical signs and mortality - At 14,28,35th day • Serology (IBD ELISA) - On 21,28Th day: • Body weights, and Bursa/body weight ratio • Histopathology • IBD PCR
Bursaplex	1st day: Bursaplex	-	
Interm./Interm. plus	9th: intermediate IBD vaccine 14thday: intermediate plus IBD vaccine	-	
Bursaplex- CH	1st day: Bursaplex	+	
Interm./Interm. Plus-CH	9th: intermediate IBD vaccine 14thday: intermediate plus IBD vaccine	+	
Challenge control	-	+	

[1] Challenge was conducted on the 16th day of age using 10^5 EID_{50} /0.1 ml of the vvIBDV strain via the oculonasal route

RESULTS

Clinical protection of immune-complex and live attenuated IBD vaccines

All vaccinated groups were protected against the vvIBDV challenge. Meanwhile, birds in the challenge control group showed 40% mortalities between day 4 and 5 post-challenge (Table 2). Upon necropsy, the challenge control group showed typical IBD lesions including hemorrhage on the thigh and breast muscle as well as bursal inflammation and enlargement. In vaccinated groups, mild bursal enlargement was noticed. no significant differences in body weights though lower body weights were observed in all groups compared to the negative control group (Table 2).

Bursa body weight ratio

Bursal body weight ratios (BB ratios) were more indicative of the clinical protection afforded by different vaccines. Both Bursaplex and intermediate/intermediate plus vaccinated non challenged groups showed higher BB ratios compared to the negative control group. The BB ratios were always significantly lower than the challenge control group especially in the Bursaplex vaccinated challenged group at 28 days of age (Figure 1).

IBDV serology in vaccinated and vaccinated challenged groups

As shown in figure 2, the ELISA antibody titers in the vaccinated nonchallenged groups were relatively higher in Bursaplex vaccinated groups at 14 and 21 day-old compared to the intermediate/intermediate plus vaccinated group. However, both groups showed no differences at 28 days of age, but the antibody titers decay was faster in the intermediate/intermediate plus vaccinated group at day 35 (figure 2A). No significant differences between the vaccination regimes upon challenge, however, the intermediate/intermediate plus vaccinated groups showed relatively higher titers (figure 2B).

Challenge virus detection and quantification

The challenge virus detection and quantification on the 28th day of age are shown in table 3. The vvIBDV was evident in the intermediate/intermediate plus vaccinated group however, no challenge virus detection was observed in the Bursaplex vaccinated challenged group. The virus was confirmed to be vvIBDV by partial sequencing of the VP2 gene (data not shown)

Histopathology

At 21 days of age, no significant bursal changes were observed in Bursaplex or Intermediate/intermediate plus vaccinated non-challenged groups except for slight B-cell proliferation of the cortical medullary cells with normal mucosal folds of the bursa in the Intermediate/intermediate plus vaccinated non-challenged group (figure 3C). Similarly, upon challenge no prominent changes while the

Bursaplex vaccinated challenged group showed lymphoproliferative follicular tissue with highly activated cortical lymphocytes (figure 3D). The challenge control showed massive degenerative changes, central necrosis and apoptosis, inflammatory cell infiltration, and aggregations of necrotic and apoptotic (Figure 3F).

By the 28th day of age, similar bursal appearance in the vaccinated non challenged groups were observed (Figure 4 B and C). In the vaccinated challenged groups, the Bursaplex vaccinated group showed a well-formed healthy epithelial lining of the pica and characteristic lymphoproliferative follicular tissue, and the cortical lymphocytes were highly activated and appeared with compacted deep basophilic nuclei and scanty cytoplasm (Figure 4D). The Bursa of intermediate/intermediate plus vaccinated challenged chickens' group is showed multiple immune-reactive lymphoid follicles and mild thickening of the interstitial connective tissue by fibroblastic proliferation (Figure 4E). The challenge control group bursae showed medullary massive central necrosis, apoptosis, and sometimes heterophilic infiltration. The cortical layer appeared completely necrotic with the disappearance of almost all the lymphoid cells (Figure 4F).

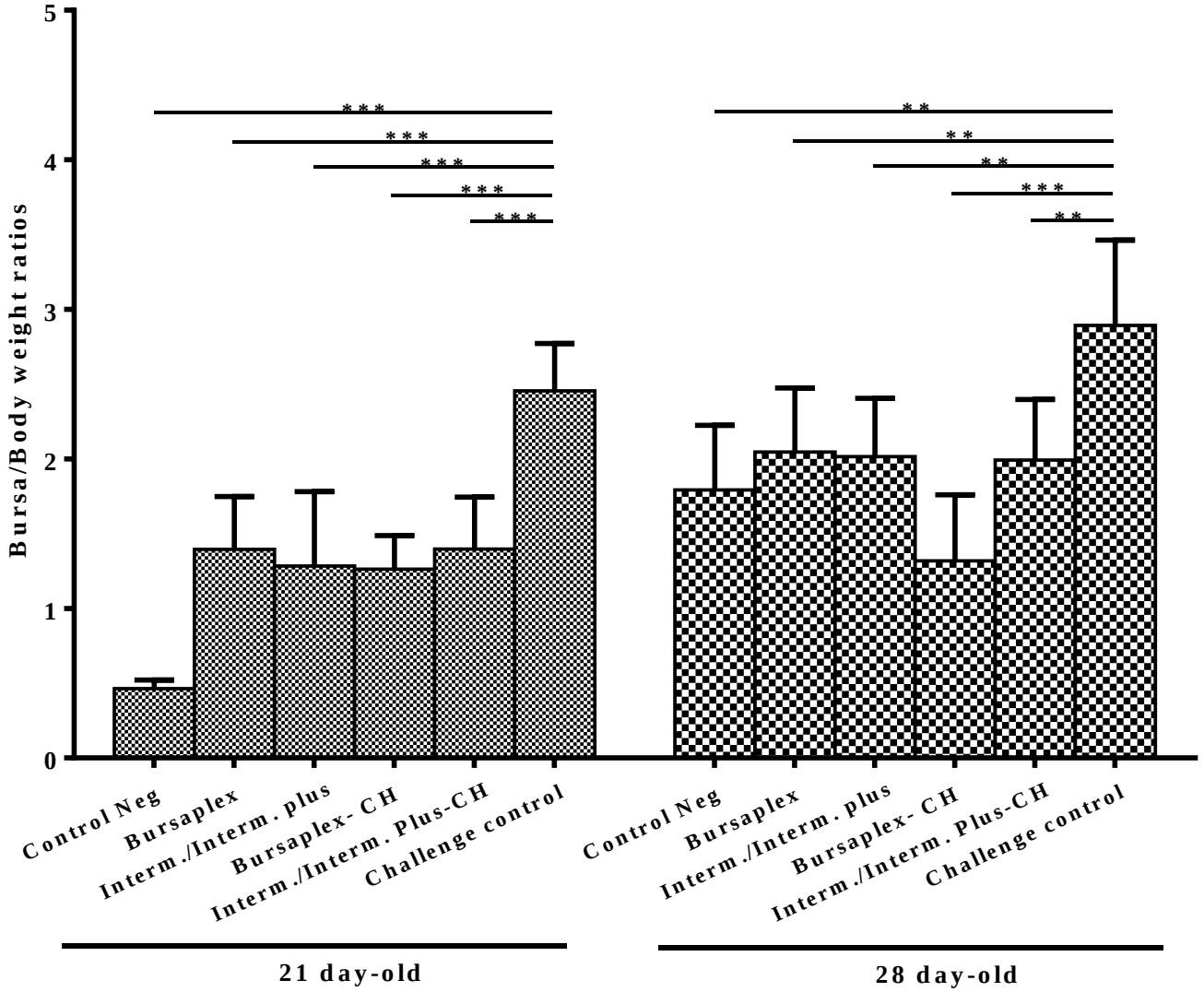


Figure 1. Bursa body weight ratios in different experimental groups at 21 and 28 day-old**.** The stars indicate significant differences at *≤0.05, **≤0.001, ***≤0.0001

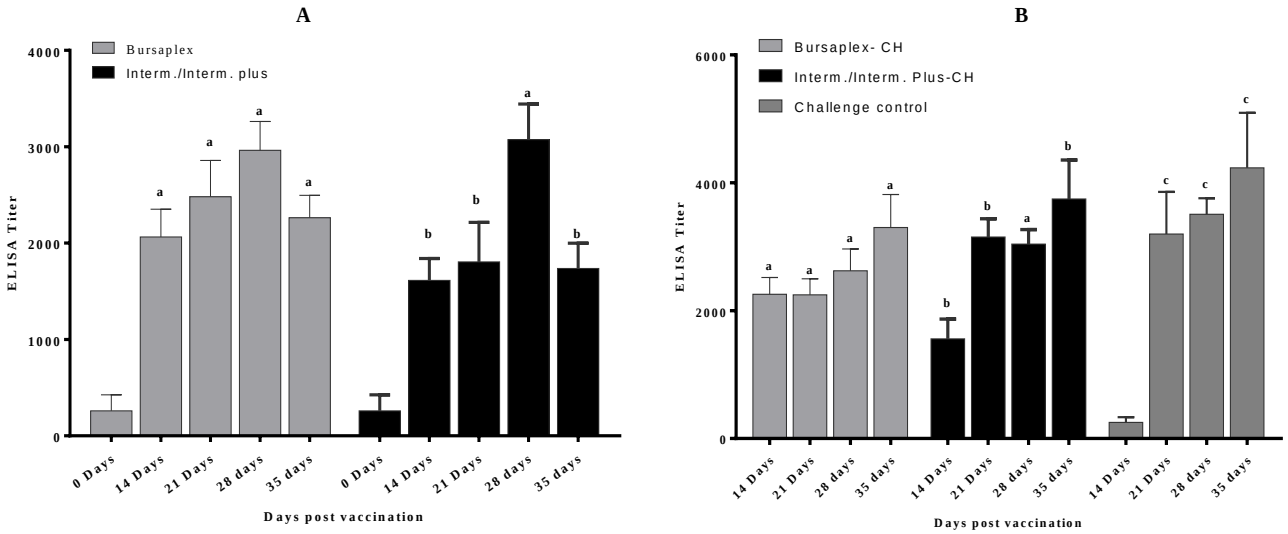


Figure 2. ELISA antibody titers in vaccinated non challenged (A) and vaccinated challenged groups (B) at 14, 21, 28, and 35 day-old. Bars with different small letters at the same time point are significantly different ($p \leq 0.05$)

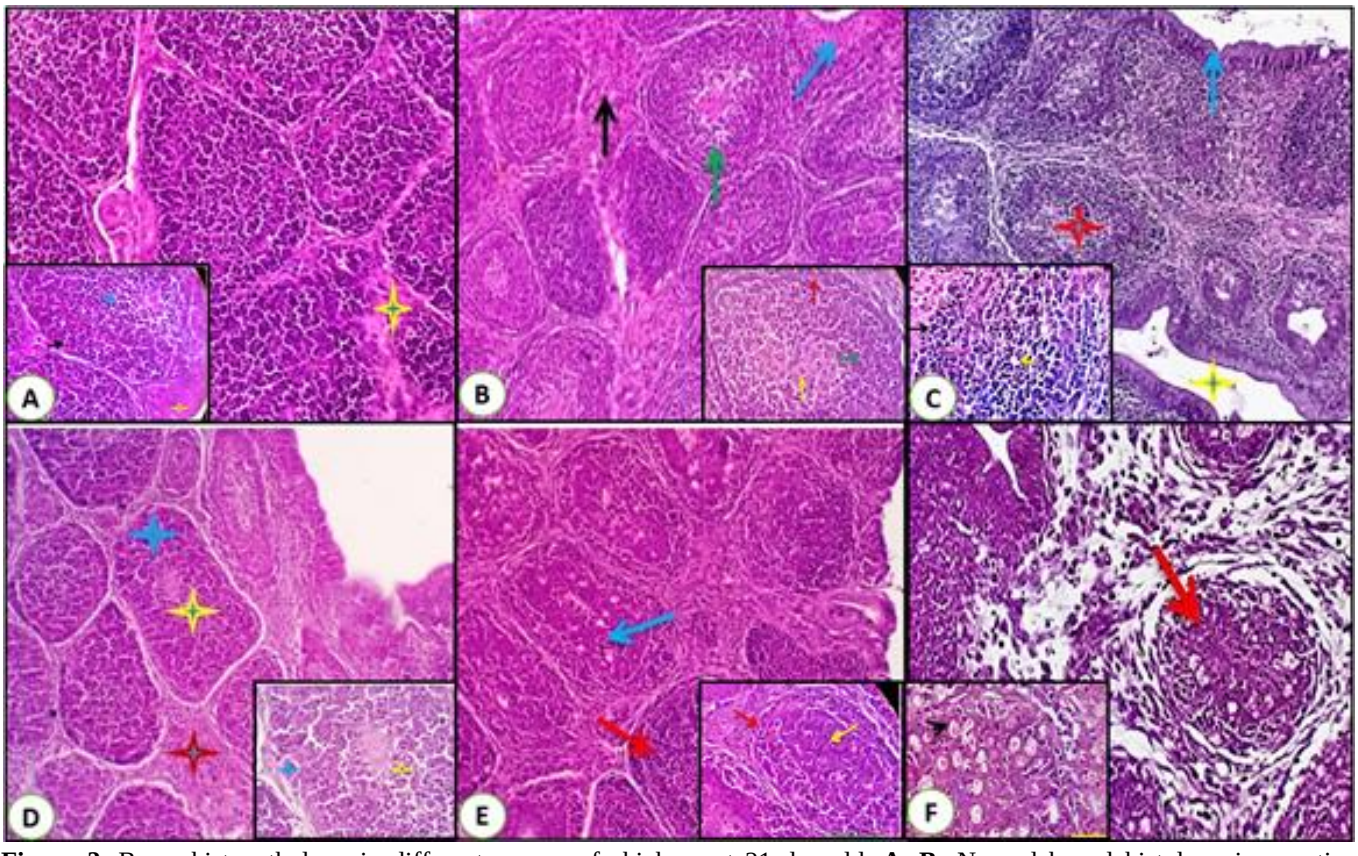


Figure 3. Bursa histopathology in different groups of chickens at 21 day-old. **A, B:** Normal bursal histology in negative control and Bursaplex vaccinated non-challenged group. **C**: Intermediate/intermediate plus vaccinated non challenged group showing slight B-cell proliferation of the cortical medullary cells (red and yellow arrow) with normal mucosal folds of the bursa (blue arrow). **D:** Bursaplex vaccinated challenged group showing lymphoproliferative follicular tissue with prominent central medullary lymphocytes (yellow stars) and the cortical lymphocytes were highly activated (dark blue stars). **E:** Intermediate/intermediate plus vaccinated challenged group showing normal bursal folds and immune-reactive lymphoid follicles. **F:** Challenge control showing degenerative changes (red arrows), massive central necrosis and apoptosis, inflammatory cells infiltration with the presence of aggregates of necrotic and apoptotic debris (black arrowhead).

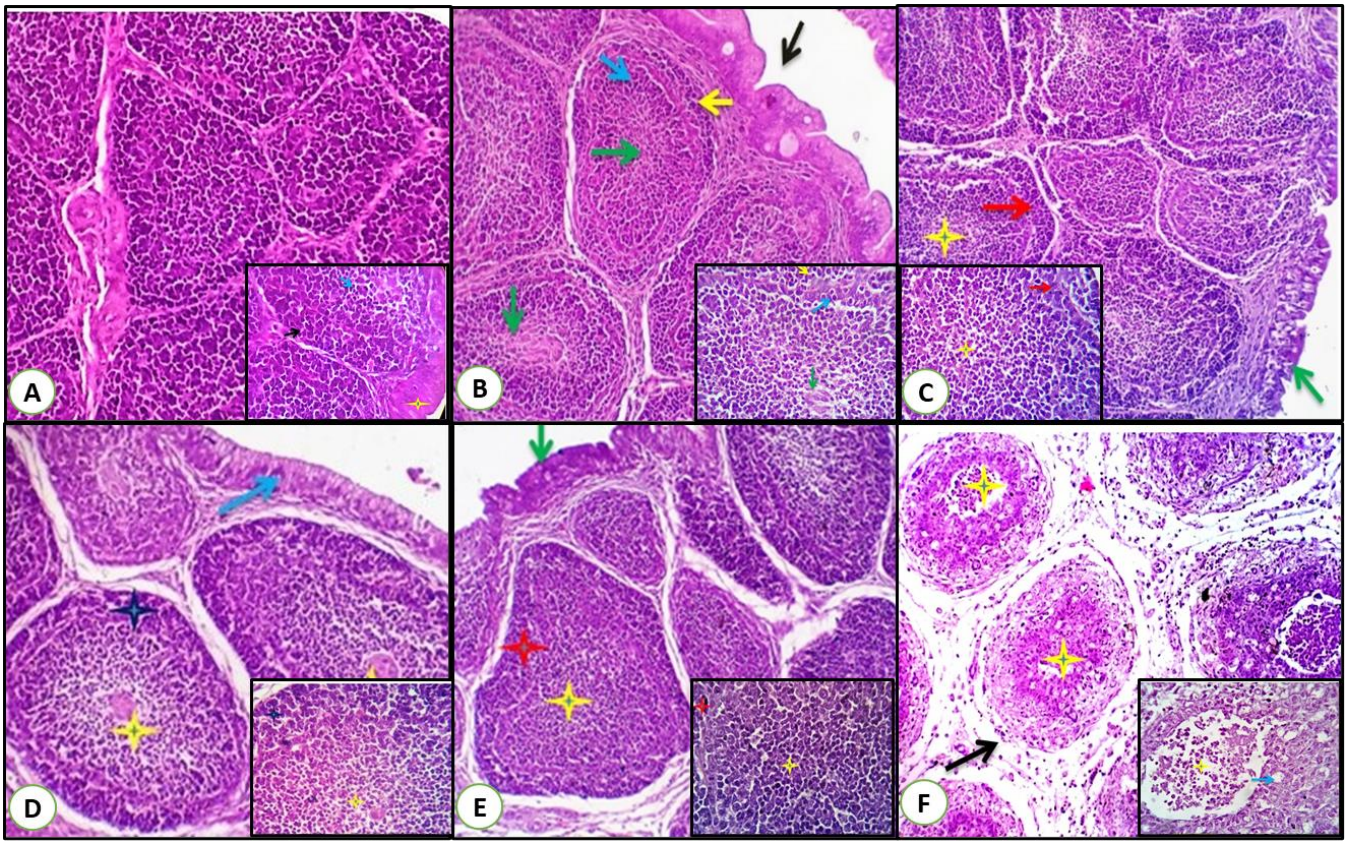


Figure 4. Bursa histopathology in different groups at 28 day-old. A: normal bursal histology in the negative control group. **B:** Bursaplex vaccinated non challenged group showing mucosal folds clear margins of the follicles, a layer of undifferentiated epithelial cells occupied the periphery of the medulla (green arrows). Both cortical (blue and yellow arrows) and medullary cellular contents (green arrows) are moderately reactive with closely backed small and large lymphocytes. **C:** intermediate/intermediate plus vaccinated non challenged group showing large sized follicles with proliferation of the cortical (red arrows) and medullary cells (yellow stars), the medullary centers appear slightly pale and crowded by large proliferating B-cells (yellow stars). **D:** Bursaplex vaccinated challenged group showed healthy epithelial lining of the pica (blue arrow) and lymphoproliferative follicular tissue (yellow stars), highly activated cortical lymphocytes with deep basophilic nuclei, and scanty cytoplasm (dark blue stars). **E:** intermediate/intermediate plus vaccinated challenged group showing normal bursal folds (green arrows), multiple immune-reactive lymphoid follicles (red stars and yellow stars), and mild thickening of the interstitial connective tissue by fibroblastic proliferation (green star). **F:** challenge control group showing massive medullary necrosis, apoptosis, heterophilic infiltration with central aggregates of necrotic and apoptotic debris (yellow stars and black arrows), the cortical layer appeared completely necrotic with the complete lymphoid cell depletion (blue stars).

Table 2. Daily mortalities and protection percent in different vaccinated chickens against vvIBDV challenge

Challenge	Groups	Daily Mortalities (days post-challenge)						Protection %
		3	4	5	Live	Dead	Total	
No	Negative control	-	-	-	20	0	20	100
	Bursaplex[1]	0	0	0	20	0	20	100
	Interm. /Interm. Plus[2]	0	0	0	20	0	20	100
Yes	Bursaplex- CH[1]	0	0	0	20	0	20	100
	Interm. /Interm. Plus-CH[2]	0	0	0	20	0	20	100
	Challenge control	0	5	3	12	8	20	40

[1]Vaccination schedule: 1st day: Bursaplex [2]Vaccination schedule: 9th: intermediate IBD vaccine- 14thday: intermediate plus IBD vaccine

Table 3. Body weights in different experimental groups at 21 and 28 day-old chickens

Age		Control Neg	Non challenged		Challenged		Challenge control
			Bursaplex	Interm./Interm. plus	Bursaplex- CH	Interm./Interm. Plus-CH	
21 days	Mean	128.86	131.89	126.87	124.35	123.12	122.68
	SD	9.07	19.17	13.60	8.40	13.03	12.85
28 days	Mean	175.93	157.94	158.06	157.30	162.84	144.84
	SD	35.61	15.37	8.10	15.72	10.77	5.88

Table 4. Very virulent and vaccinal IBDV shedding detection at 28 day-old in vaccinated and vaccinated-challenged groups

Group	Sample	IBDV virus detection by RT-PCR		
		Very virulent IBDV	Vaccinal IBDV	Result
Bursaplex	5 pooled (bursa)	-	28,6	vvIBDV: NEG vacc.IBDV: POS
Interm./Interm. plus		-	24,3	vvIBDV: NEG vacc.IBDV: POS
Bursaplex- CH		-	30,8	vvIBDV: NEG vacc.IBDV: POS
Interm./Interm. Plus-CH		24,0	-	vvIBDV: POS vacc.IBDV: NEG
Challenge Control		25,6	-	vvIBDV: POS vacc.IBDV: NEG
Negative control		-	-	IBDV: NEG

DISCUSSION

In this study 2 vaccination schedules against vvIBDV were compared. Two live attenuated intermediate and intermediate plus vaccines at 9 and 14th day-old, respectively versus an immune-complex vaccine (Bursaplex) at day-old. The performance, bursal gross lesions, antibody immune response, and bursal histopathology were evaluated in vaccinated non challenged and vaccinated challenged birds. All vaccinated groups were protected against the vvIBDV challenge compared to 40% mortality in the challenge control group. Though the challenge control showed lower body weight, however, these differences were not statistically significant.

Both the immune-complex and live attenuated IBD vaccine groups showed higher BB ratios compared to the negative control group. However, on the 28th day of age, the BB ratio in the immune-complex vaccinated challenged group was significantly lower than the live attenuated group. Immune-complex IBDV vaccines have shown lower BB ratios especially when administered via in-ovo vaccination compared to live vaccine (Le Gros et al., 2009; Roh et al., 2016). Though intermediate plus vaccines contain more pathogenic strains (Sedeik et al., 2019), however, the BB ratios did not significantly differ from those of the immune-complex vaccine that might be attributed to the use of 2 doses of intermediate vaccine on the 9th day (Roh et al., 2016), and the intermediate plus vaccine at 14th day allowing for better bursal recovery than using intermediate plus vaccine alone (Aihara et al., 2015; Lupini et al., 2020).

Serologically, the ELISA antibody titers follow up in vaccinated non challenged groups indicated that Bursaplex vaccinated groups antibody titers were significantly higher except at 28th day of age, where the live attenuated vaccine was significantly higher. Similar results were previously reported (Zorman Rojs et al., 2011), where using an ELISA kit utilizing a bursa-derived antigen (e.g. Proflok plus IBD Ab kit, Synbiotics). After the challenge, the intermediate/intermediate plus vaccinated challenged group has significantly higher antibody titers at 21st and 35th days of age indicating more challenge virus replication compared to the immune-complex group (Abaza et al., 2020). These results were confirmed by the challenge virus detection and quantification on the 28th day where the vvIBDV was detected with high cycle thresholds (ct) value using the real-time RT-PCR indicating high shedding titers of the challenge virus (Techera et al., 2019). Conversely, a recent study showed that both immune-complex and live attenuated IBD vaccines did not induce sterile immunity as the challenge virus was detected in both groups (Ivanet al., 2005; Prandiniet al., 2016).

The immune-complex vaccinated challenged group developed milder bursal histopathology but the difference to the intermediate/intermediate plus vaccination regime was not significant. Previously, IBD live-vaccinated birds

displayed comparable histopathology to the challenge control group (Prandini et al., 2016; Kurukulasuriya et al., 2017), however, the alleviated bursal damage in the intermediate/intermediate plus vaccinated group may be attributed to the adjacent dosing regimen followed in the current study (i.e. 9th and 14th day of age). It is worthy to note that the current study was conducted in specific pathogen free chickens in the absence of maternally derived antibodies. Previous studies have shown that the immunocomplex vaccine showed poor immune response with even negative ELISA titers at 3 weeks of age due to the remaining neutralization antibody activity of the vaccine (Bose et al., 2016; Sedeik et al., 2019).

In conclusion, the use of either immune-complex vaccine at day-old or early vaccination with intermediate and intermediate plus live attenuated IBD vaccines induced protective antibody titers and both programs were clinically protective against an early vvIBDV challenge. However, the immune-complex vaccine induced sterile immunity as the challenge virus was not detected on the 28th day of age. Similar programs testing in commercial broilers in the presence of maternal derived IBD antibodies to simulate field conditions are required.

REFERENCES

Abaza MA, Elboraay EM, Saad AE and Zayan KA (2020). Assessment of the role of intracloacal inoculation of live infectious bursal disease vaccine in breaking through maternally derived antibodies. Avian Pathology, 1-8. DOI: https://www.doi.org/10.1080/03079457.2020.1796925

Aihara N, Horiuchi N, Hikichi N, Ochiai M, Hosoda Y, Ishikawa Y, Shimazaki Y and Oishi K (2015). Immunoreactivity and morphological changes of bursal follicles in chickens infected with vaccine or wild-type strains of the infectious bursal disease virus. Journal of Veterinary Medical Science. 77 (8): 913-918. DOI: https://www.doi.org/10.1292/jvms.14-0599

Alkie TN and Rautenschlein S (2016). Infectious bursal disease virus in poultry: Current status and future prospects. Veterinary Medicine: Research and Reports, 7: 9-18. DOI: https://www.doi.org/10.2147/VMRR.S68905

Bose RK, Hossain KM, Sil BK, Taimur M, Pugliese C and Franci O (2016). Comparative seroevaluation of live and killed gumboro vaccine in broilers. Italian Journal of Animal Science. 2 (2): 157-162. DOI: https://www.doi.org/10.4081/ijas.2003.157

Camilotti E, Moraes L, Furian T, Borges K, Moraes H and Salle C (2016). Infectious bursal disease: Pathogenicity and immunogenicity of vaccines. Brazilian Journal of Poultry Science, 18: 303-308. DOI: https://www.doi.org/10.1590/1806-9061-2015-0148

Delmas B, Attoui H, Ghosh S, Malik YS, Mundt E, Vakharia VN and Ictv Report C (2019). Ictv virus taxonomy profile: Birnaviridae. Journal of General Virology, 100 (1): 5-6. DOI: https://www.doi.org/10.1099/jgv.0.001185

Dey S, Pathak DC, Ramamurthy N, Maity HK and Chellappa MM (2019). Infectious bursal disease virus in chickens: Prevalence, impact, and management strategies. Veterinary Medicine: Research and Reports, 10: 85-97. DOI: https://www.doi.org/10.2147/VMRR.S185159

Eterradossi N and Saif YM (2016). Infectious bursal disease (gumboro disease). In: Manual of diagnostic tests and vaccines for terrestrial animals. World Organisation for Animal Health, Chapter 3.3.12.

Eterradossi N and Saif YM (2020). Infectious bursal disease. In: Diseases of poultry. 14, Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, Wit Sd, Grimes T, Johnson D, Kromm M et al. Hoboken, NJ 07030, USA. John Wiley & Sons, Inc, 257-283. https://www.doi.org/10.1002/9781119371199.ch7

Ewies SS, Ali A, Tamam SM and Madbouly HM (2017). Molecular characterization of Newcastle disease virus (genotype vii) from broiler chickens in Egypt. Beni-Suef University Journal of Basic and Applied Sciences, 6 (3): 232-237. DOI: https://www.doi.org/10.1016/j.bjbas.2017.04.004

Ingrao F, Rauw F, Lambrecht B and Van den Berg T (2013). Infectious bursal disease: A complex host-pathogen interaction. Developmental & Comparative Immunology, 41 (3): 429-438. DOI: https://www.doi.org/10.1016/j.dci.2013.03.017

Ivan J, Velhner M, Ursu K, German P, Mato T, Dren CN and Meszaros J (2005). Delayed vaccine virus replication in chickens vaccinated subcutaneously with an immune complex infectious bursal disease vaccine: Quantification of vaccine virus by real-time polymerase chain reaction. Canadian Journal of Veterinary Research, 69 (2): 135-142.

Kurukulasuriya S, Ahmed KA, Ojkic D, Gunawardana T, Goonewardene K, Gupta A, Chow-Lockerbie B, Popowich S, Willson P, Tikoo SK et al. (2017). Modified live infectious bursal disease virus (IBDV) vaccine delays infection of neonatal broiler chickens with variant IBDV compared to turkey herpesvirus (HVT)-IBDV vectored vaccine. Vaccine, 35 (6): 882-888. DOI: https://www.doi.org/10.1016/j.vaccine.2017.01.005

Le Gros FX, Dancer A, Giacomini C, Pizzoni L, Bublot M, Graziani M and Prandini F (2009). Field efficacy trial of a novel hvt-ibd vector vaccine for 1-day-old broilers. Vaccine. 27 (4): 592-596. DOI: https://www.doi.org/10.1016/j.vaccine.2008.10.094

Lupini C, Quaglia G, Mescolini G, Russo E, Salaroli R, Forni M, Boldini S and Catelli E (2020). Alteration of immunological parameters in infectious bronchitis vaccinated-specific pathogen-free broilers after the use of different infectious bursal disease vaccines. Poultry Science, 99 (9): 4351-4359. DOI: https://www.doi.org/10.1016/j.psj.2020.05.054

Muller H, Mundt E, Eterradossi N and Islam MR (2012). Current status of vaccines against infectious bursal disease. Avian Pathology, 41 (2): 133-139. DOI: https://www.doi.org/10.1080/03079457.2012.661403

Perozo F, Villegas AP, Fernandez R, Cruz J and Pritchard N (2009). Efficacy of single dose recombinant herpesvirus of

turkey infectious bursal disease virus (IBDV) vaccination against a variant IBDV strain. Avian Diseases, 53(4): 624-628. DOI: https://www.doi.org/10.1637/8687-31009RESNOTE.1

Prandini F, Simon B, Jung A, Poppel M, Lemiere S and Rautenschlein S (2016). Comparison of infectious bursal disease live vaccines and a HVT-IBD vector vaccine and their effects on the immune system of commercial layer pullets. Avian Pathology, 45 (1): 114-125. DOI: https://www.doi.org/10.1080/03079457.2015.1127891

Rautenschlein S, Yeh HY and Sharma JM (2003). Comparative immunopathogenesis of mild, intermediate, and virulent strains of classic infectious bursal disease virus. Avian Diseases, 47 (1): 66-78. DOI: https://www.doi.org/10.1637/0005-2086(2003)047[0066:CIOMIA]2.0.CO;2

Roh JH, Kang M, Wei B, Yoon RH, Seo HS, Bahng JY, Kwon JT, Cha SY and Jang HK (2016). Efficacy of HVT-IBD vector vaccine compared to attenuated live vaccine using in-ovo vaccination against a Korean very virulent IBDV in commercial broiler chickens. Poultry Science, 95 (5): 1020-1024. DOI: https://www.doi.org/10.3382/ps/pew042

Schat KA, Martins NR, O'Connell PH and Piepenbrink MS (2011). Immune complex vaccines for chicken infectious anemia virus. Avian Diseases, 55 (1): 90-96. DOI: https://www.doi.org/10.1637/9347-032910-ResNote.1

Sedeik ME, El-Shall NA, Awad AM, Abd El-Hack ME, Alowaimer AN and Swelum AA (2019). Comparative evaluation of HVT-IBD vector, immune complex, and live IBD vaccines against vvIBDV in commercial broiler chickens with high maternally derived antibodies. Animals (Basel), 9 (3). DOI: https://www.doi.org/10.3390/ani9030072

Sharma JM, Kim IJ, Rautenschlein S and Yeh HY (2000). Infectious bursal disease virus of chickens: Pathogenesis and immunosuppression. Developmental & Comparative Immunology, 24 (2-3): 223-235. DOI: https://www.doi.org/10.1016/s0145-305x(99)00074-9

Shehata AA, Sedeik ME, Elbestawy AR, Zain El-Abideen MA, Ibrahim HH, Kilany WH and Ali A (2019). Co-infections, genetic, and antigenic relatedness of avian influenza h5n8 and h5n1 viruses in domestic and wild birds in Egypt. Poultry Science, 98 (6): 2371-2379. DOI: https://www.doi.org/10.3382/ps/pez011

Sivanandan V and Maheswaran SK (1980). Immune profile of infectious bursal disease: I. Effect of infectious bursal disease virus on peripheral blood t and b lymphocytes of chickens. Avian Diseases, DOI: 24 (3): 715-725.

Stoute ST, Jackwood DJ, Sommer-Wagner SE, Cooper GL, Anderson ML, Woolcock PR, Bickford AA, Senties-Cue CG and Charlton BR (2009). The diagnosis of very virulent infectious bursal disease in California pullets. Avian Diseases, 53 (2): 321-326. DOI: https://www.doi.org/10.1637/8684-030909-Case.1

Suvarna K, Layton C and Bancroft J (2018). Bancroft's theory and practice of histological techniques.8th, London, United Kingdom. Churchill Livingstone Elsevier.

Techera C, Tomas G, Panzera Y, Banda A, Perbolianachis P, Perez R and Marandino A (2019). Development of real-time PCR assays for single and simultaneous detection of infectious bursal disease virus and chicken anemia virus. Molecular and Cellular Probes, 43: 58-63. DOI: https://www.doi.org/10.1016/j.mcp.2018.11.004

Zorman Rojs O, Krapez U, Slavec B, Jursic-Cizerl R and Poljanec T (2011). Field efficacy of different vaccines against infectious bursal disease in broiler flocks. Acta Veterinaria Hungarica, 59 (3): 385-398. DOI: https://www.doi.org/10.1556/AVet.2011.016

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 565-570, December 25, 2020

Research Paper, PII: S2322455X2000064-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.64

Reproductive Performance of Koekoek Chickens at Different Levels of Feed Restrictions

Setsumi Motsoene Molapo [1]*, Edward Webb[2], Motselisi Aloycia Mahlehla[1], Thato Chabeli[1], and Paseka Kompi[1]

[1] *Department of Animal Science, National University of Lesotho, P.O. Roma 180, Lesotho*
[2] *Department of Animal and Wildlife Sciences, University of Pretoria, Pretoria 0002, South Africa*

*Corresponding author's Email: setsomimolapo@gmail.com, ORCID: 0000-0002-4426-8901

Received: 07 Oct. 2020
Accepted: 18 Dec. 2020

ABSTRACT

The objective of the present study was to determine the impact of the feeding levels on the reproductive characteristics of Koekoek chickens. A total of 270 Koekoek chickens were randomly assigned to 4 feeding level treatments in a completely randomized design. The four feeding level treatments were fully fed during the rearing and laying phase (AA), fully fed during the rearing phase and restricted feeding during the laying phase (AR), restricted feeding during the rearing phase and fully fed during the laying phase (RA), and restricted both during the rearing and laying phase (RR). The General Linear Model procedure (SPSS software, version 17) was used to analyze the data set. The pubic bone measurements were 23.6, 25.1, 16.1, and 15.1 mm for chickens that received AA, AR, RA, and RR treatments, respectively, at 18 weeks of age. At 32 weeks of age, chickens given AA and RA treatments had wider pubic bones than chickens given AR and RR treatments. Combined ova and oviduct weights were higher in the fully fed chickens at 18 weeks of age. Koekoek chickens in AA treatment had the highest average egg production. Chickens given AR treatment had lower average egg weights than those given AA, AR, and RR treatments. Chickens treated with AA and AR reached puberty earlier than those that were treated with the RA and RR treatments. The eggs produced by chickens given RR treatment had a higher average hatching percentage. The lowest percentage of hatches was observed in chickens that were fed *ad libitum* during the rearing phase. In conclusion, the feed restriction only during the rearing phase improved the reproduction performance of Koekoek chickens.

Keywords: Egg weight, Fully fed, Hatchability, Koekoek, Laying percentage, Oviduct, Pubic bone, Restricted

INTRODUCTION

Feeding is one of the greatest determinants required for higher egg production. The high costs of chicken feed are making it impossible for the poor resource farmers in rural areas of Lesotho to keep laying chickens. One of the strategies to reduce high feeding costs is to use restricted feeding**.** Salih et al. (2016) believed that periodic restriction of the daily feed to simulate compensatory growth was a means of reducing feed cost. Feed restriction was used to control the development of chickens in order to reduce the incidence of metabolic diseases and enhance feed efficiency (Trocino et al., 2020). In addition, Adegbenro et al. (2020) indicated that feed restriction was the strategy aimed at reducing the growth and metabolic rate and hence the delay in sexual maturity of chickens during the growing phase. This consequently increased body weight and production without negatively impacting welfare and health. Moreover, Simeneh (2019) confirmed that unrestricted feeding of laying hens led to waste of energy, which promoted the unnecessary build-up of abdominal fat and predisposed layers to heat stress.

Yildiz et al. (2006) considered feed restriction as a strategy to reduce oviduct development. Crounch et al. (2002) also reported that feed restriction during the rearing phase could result in heavier mature ova. The higher hatchability was recorded in the treatment with restricted feed in a study on turkeys (Crounch et al., 2002). However, Melnychuk et al. (2004) reported heavy oviducts in chickens that were fed *ad libitum*. Crounch et al. (2002) also found similar results although the study was performed on turkeys. Regarding ovary development, some studies indicated a greater weight in the *ad libitum* fed chickens (Melnychuk et al., 2004; Renema et al., 1999). On the other hand, the obtained results of a study performed by Evrim and Kaya (2014) showed similar laying production between quails fed differently prior to sexual maturity. In a study conducted on quails, it was indicated that fertility and hatchability were not affected by the feeding regime (Evrim and Kaya, 2014).

To cite this paper: Molapo Motsoene S, Webb E, Aloycia Mahlehla M, Chabeli Th, and Kompi P (2020). Reproductive Performance of Koekoek Chickens at Different Levels of Feed Restrictions. *J. World Poult. Res.*, 10 (4): 565-570. DOI: https://dx.doi.org/10.36380/jwpr.2020.64

Since the Koekoek chickens were introduced in Lesotho, insufficient or no scientific studies have been conducted on their feeding management. Therefore, it is important to determine the feeding level that aims to maximize egg production and hatchability at affordable feed costs. The present study was, therefore, carried out to investigate the effect of restricted feeding on the laying and hatching performance of Koekoek chickens.

MATERIALS AND METHODS

Ethical approval

The Research and Ethics Committee in the Department of Animal Science at the National University of Lesotho approved this study based on international animal welfare standards for the use of animals in conducting research.

Animals management and experimental design

The present study was carried out at the National University of Lesotho situated in Roma, Lesotho, an experimental farm of the Faculty of Agriculture. Chickens were bought and fed commercial feeds at eight weeks of age. Upon arrival, they were given a stresspack (water soluble vitamin and electrolyte supplement) to reduce travel stress, which could lead to death. Chickens were reared under the deep litter system. Each pen was equipped with three wooden nests measuring 40×40×40 cm. From 8 to 18 weeks chickens ate pullet grower, then from 19 to 32 weeks, they were fed laying mash bought from the local commercial feed manufacturer. Chickens were given water without restriction. A completely randomized design of four feeding level treatments was used. The four feeding level treatments were AA, AR, RA, and RR. Each treatment had 10 hens and one rooster. Each treatment was replicated 7 times, except for the RR treatment, which was replicated 6 times, which means there were 270 hens and 27 roosters. At day 130 and 32 weeks (224 days) of age, 7 Koekoek hens per treatment were killed by cervical dislocation. The chickens were starved for 24 hours prior to slaughter. The ovaries and oviducts were collected and weighed. The oviducts were emptied of the contents. The ovaries were examined for follicular development. The diameter of the pubic bones was measured. The eggs were collected daily and an average laying percentage was calculated for each week throughout the study period. The egg weights were recorded after 25 weeks, at which the majority of chickens began to lay in all replicates. The ages at which Koekoek chickens first reached puberty (point of lay), 20%, 50%, and greater or equal to 80% egg productions were recorded. A sample of three eggs weighing between 50 and 55 gram (g) from each replicate for all treatments and less than eight days old was removed and placed in a model SH680 fully automatic digital egg incubator made in South Africa. During the incubation period, the eggs were not turned for the first three days. During days 4-18, egg turning was done three times a day. On day 18, the eggs were removed from trays and placed into the hatching trays until hatching time. The incubator was not disturbed for the last three days of incubation. The chickens were removed from the incubator in the morning after 22 days. The hatching percentage of the eggs was calculated as follows:

Hatching percent = Total number of eggs hatched × 100 / Total number of eggs incubated

Statistical analysis

The data were analyzed using the statistical package for social sciences (SPSS version 17). General Linear Model (GLM) procedure was used to analyze the effect of feed restriction on the reproductive performance of Koekoek chickens.

RESULTS

Ova and oviducts weights

The weight of the ova and oviduct in pullets fully fed during the rearing phase (AA and AR) was 64.6% higher than that in the restricted feeding treatments (RA and RR) as stated in table 1. At 32 weeks of age, Koekoek hens that were fed unrestrictedly during the laying phase (AA and RA) had similar ($p<0.05$) ova weights, compared to those that were fed restrictedly during the laying phase (AR and RR). Despite the insignificant differences in ova weight between chickens that were subjected to different feeding levels, the ova weights of restricted feeding chickens (RR) were higher ($p>0.05$) than those of hens that were in the AA, AR, and RA treatments by 3.4%, 2.5%, and 4.7%, respectively. This suggests that restricted feeding during the laying phase stimulated the ova production in Koekoek chickens.

Pubic bones width

At puberty (18 weeks), fully fed Koekoek chickens had higher ($p<0.05$) pubic bone measurements than those under feed restriction (Table 1). On average, the pubic bone widths of chickens that were fully fed were 35.9% greater ($p<0.05$) than the average pubic bone widths of chickens that were subjected to restricted feeding at 18

weeks of age. During the laying phase (32 weeks), Koekoek chickens that were fully fed during the laying phase had larger ($p<0.05$) pubic bone widths than those that were fed restrictedly. Koekoek chickens in the RA treatment had a faster pubic bone width growth of 66.9%, followed by chickens in the RR treatment with 66.2%. The lowest in pubic bone width growth was observed in chickens assigned to the AR treatment (40.5%), while chickens assigned the AA treatment (51.8%) ranked the third position between 18 and 32 weeks. These results revealed that restricted feeding during the rearing phase promoted the development of the pubic bones more than full feeding during the same period.

Table 1. Pubic bones, ova, and oviduct growth of Koekoek chickens subjected to different levels of feeding treatments

		Treatments				
Age	**Variables**	**AA**	**AR**	**RA**	**RR**	**S.E**
18 weeks	Pubic bone (mm)	23.6[a]	25.1[a]	16.1[b]	15.1[b]	0.66
	Ova and oviducts (g)	16.0[a]	15.4[a]	5.5[b]	5.5[b]	0.39
32 weeks	Pubic bone (mm)	48.9[a]	43.9[b]	48.8[a]	44.6[b]	0.50
	Ova weight (g)	46.1	46.6	45.5	47.8	0.78
	Oviduct weight (g)	49.1	48.1	52.0	48.0	0.80

[a,b:] Means within a row with no common superscript differ significantly ($p<0.05$), S.E: Standard Error. AA: Full feeding during rearing and laying, AR: Full feeding during rearing and restricted during laying, RA: Restricted feeding during rearing and full feeding during laying, RR: Restricted feeding during rearing and laying.

Laying performance

Koekoek chickens that were subjected to different feeding levels had ($p<0.05$) different laying percentages between 18 and 32 weeks of age (Figure 1). Koekoek chickens that were fully fed during the rearing phase (AA and AR) had a significantly ($p<0.05$) higher laying percentage between 19 and 21 weeks old, compared to those with restricted feeding (RA and RR). Koekoek chickens that were fully fed throughout the study (AA) had the highest laying percentage, followed by those that were only fed restrictedly during the rearing phase (RA). Koekoek chickens that were only fed restrictedly during the rearing phase (RA) responded positively to the fully fed diet after four weeks. The positive response of RA treatment can be confirmed by the similar laying percentages of chickens that were in the AA and RA treatments from week 25. On average, chickens that were fully fed during the rearing and laying phase (AA) had higher ($p<0.05$) laying percentage.

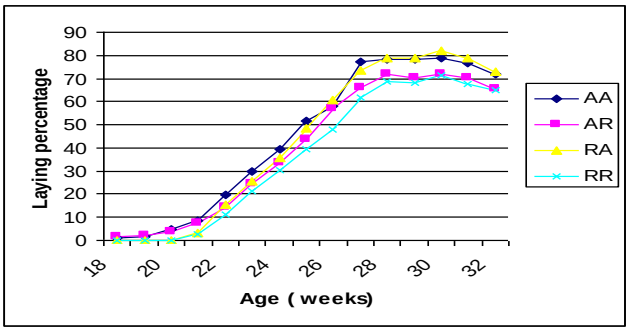


Figure 1. The laying percentage of Koekoek chickens subjected to different feeding levels. AA: Full feeding during rearing and laying, AR: Full feeding during rearing and restricted during laying, RA: Restricted feeding during rearing and full feeding during laying, RR: Restricted during rearing and laying,

Egg weights

The egg weight of chickens that received different feeding level treatments significantly differed ($p<0.05$) throughout the experiment, except at the ages of 27 and 28 weeks (Table 2). At 25 weeks of age, the egg weights of Koekoek chickens that were fed restrictedly for the entire study (RR) were 27.4%, 22.6%, and 30.4% lower ($p<0.05$) than the egg weights of chickens that were under the AA, RA, and AR treatments, respectively. On average, chickens that were fully fed during the laying phase (AA and RA) produced eggs of higher weights ($p<0.05$) than those that were exposed to restricted feeding (AR and RR) during the same phase.

Different egg production stages

The results on how chickens that were either full-fed or restricted fed performed in terms of age at puberty, 20%, 50%, and 80% egg production are presented in table 3. The results revealed that restricted feeding affected the number of days to reach different egg production phases.

Hatchability

Eggs produced by Koekoek chickens subjected to feed restrictions during the laying period (AR and RR) hatched higher ($p<0.05$) than the eggs from chickens that were unrestrictedly fed during the laying phase (AA and RA) at 28 weeks of age (Table 4). Restricted feeding during the laying phase increased the hatchability of the eggs in Koekoek chickens. The hatching percentages of eggs in the RR treatment were 13.5%, 8.9%, and 10.5% higher ($p<0.05$) than in chickens assigned to the AA, AR,

and RA treatments, respectively. Hatchability increased with age since the hatching percentages of eggs produced from chickens under the AA, AR, RA, and RR treatments increased by 23%, 10.9%, 21.4%, and 8.9%, respectively. Generally, restricting feed during the laying period could increase hatchability, compared to feeding completely during the laying phase. This meant that the hatching percentage during the laying phase is negatively related to the feed intake and body weight of chickens.

Table 2. Egg weights of Koekoek chickens subjected to different feeding level treatments

Treatments / Age (Weeks)	AA	AR	RA	RR	S.E
25	37.5^{a}	35.6^{a}	39.6^{a}	27.6^{b}	1.06
26	45.5^{a}	39.7^{b}	45.0^{a}	39.0^{b}	0.73
27	46.3	45.7	47.2	46.9	0.32
28	46.0	45.0	47.1	46.9	0.42
29	48.4^{a}	46.3^{b}	48.1^{a}	46.7^{ab}	0.27
30	47.9^{ab}	47.3^{a}	49.2^{b}	48.1^{ab}	0.29
31	48.0^{a}	46.3^{b}	49.2^{a}	47.5^{ab}	0.29
32	49.2^{a}	43.9^{b}	50.2^{a}	46.9^{b}	0.70
Average	45.6^{a}	43.5^{b}	46.7^{a}	43.1^{b}	0.22

$^{a, b, c:}$ Means within a row with no common superscript differ significantly ($p<0.05$), S.E: Standard Error; AA: Full feeding during rearing and laying, AR: Full feeding during rearing and restricted during laying, RA: Restricted feeding during rearing and full feeding during laying, RR: Restricted feeding during rearing and laying.

Table 3. The number of days it takes Koekoek chickens to reach the first oviposition, 20%, 50%, and greater or equal to 80% egg-laying production

Treatments / Variables	AA	AR	RA	RR	S.E
Number of days to 1st oviposition	150.1^{a}	152.4^{a}	159.0^{b}	159.8^{b}	0.61
Number of days to 20% production	163.5^{a}	164.1^{b}	166.9^{ab}	168.3^{b}	0.79
Number of days to 50% production	174.1	172.8	175.0	176.1	0.79
Number of days to ≥ 80 % production	191.7^{a}	190.7^{a}	189.1^{a}	199.4^{b}	1.19

$^{a, b:}$ Means within a row with no common superscripts differ significantly ($p<0.05$). S.E: standard error; AA: Full feeding during rearing and laying, AR: Full feeding during rearing and restricted during laying, RA: Restricted feeding during rearing and full feeding during laying, RR: Restricted feeding during rearing and laying

Table 4. Egg hatching percentage of Koekoek chickens subjected to different feeding levels

Treatments / Age (Weeks)	AA	AR	RA	RR
28	62.1^{a}	75.7^{b}	65.7^{a}	85.0^{b}
30	75.0^{a}	89.3^{b}	78.6^{a}	92.5^{b}
32	80.7^{a}	85.0^{a}	83.6^{a}	93.3^{b}
Average	72.6^{a}	83.3^{b}	76.0^{a}	90.3^{c}

$^{a, b, c:}$ Means within a row with no common superscript differ significantly ($p<0.05$). AA: Full feeding during rearing and laying, AR: Full feeding during rearing and restricted during laying, RA: Restricted feeding during rearing and full feeding during laying, RR: Restricted feeding during rearing and laying, S.E: Standard Error

DISCUSSION

Concerning the ova weights, the results of the present study agreed with the findings of Crounch et al. (2002), who stated that the maturing ova were heavier in turkeys that were fed restrictedly during the rearing period than in those that were fed *ad libitum* in the rearing phase. Simeneh (2019) also found that limited feed at the rearing phase hindered the development of the oviducts. On the contrary, Renema et al. (1999) pointed out that restricted feeding chickens were 38% lower than *ad libitum* feeding chickens in terms of the ovary weights. In addition, Robinson et al. (2007) reported that the ovary weights were influenced by body weight and possibly by fat content. Yildiz et al. (2006) also suggested that *ad libitum*-fed chickens showed accelerated development of oviducts.

The findings of previous researchers indicated similar oviduct weights between chickens that were

subjected to different feeding regimes at sexual maturity (Tesfaye et al., 2009; Renema et al., 2007; Renema et al., 1999). Yildiz et al. (2006) also suggested that restricting feed during the rearing period significantly delayed oviduct development. Moreover, Cassy et al. (2004) and Renema et al. (2007) confirmed that chickens that were exposed to feeding restrictions during the growing period had a decreased number of large yellow follicles at first oviposition.

Crounch et al. (2002) also found that the total egg production was higher in chickens that were fully fed during the laying period. The findings of the present study tally with the results of Oyedeji et al. (2007) who reported that the egg weight was significantly higher in hens that were fed *ad libitum*, compared to those that were restricted.

Concerning egg weight, Moreira et al. (2012) also stated that it was not affected by the dietary restriction. Evrim and Kaya (2014) also found that early feed restriction did not affect the average egg weights in quails. On the other hand, Miles and Jacqueline (2000) showed that a feed restriction program would result in a slight decrease in egg size, which was less significant when the majority of eggs were in large category, which was in line with the results of the present study showing the effect after 30 weeks.

It was found that the reduction of egg production in layers that were restrictedly fed throughout the study (RR), the last group to reach one of the egg production stages, was consistent with the obtained results of a study conducted by Ezieshi et al. (2003). In a study on quails, Evrim and Kaya (2014) emphasized that feed restriction delayed the onset of laying as was observed in the findings of the present study since chickens treated with feed restrictions during the rearing phase (RA and RR) experienced delayed sexual maturity. In addition, Onagbesan et al. (2006) stated that chickens with restricted feeding would take a longer period to reach maximum egg production than chickens that were fully fed. Crounch et al. (2002) also stated that turkeys that were fed restrictedly at the rearing period (3-16 weeks) had a significantly higher egg production than those that were fully fed during the growing period.

In support of these results, Simeneh (2019) stated that early feed restriction reduced overall embryonic mortality. Crounch et al. (2002) also indicated that turkeys that were shifted from restricted feeding during the rearing phase to *ad libitum* feeding during the laying phase had a significantly higher embryonic mortality, and thus a lower hatching percentage compared to the other treatments. Evrim and Kaya (2014) also reported that mortality in fully fed quails increased by 56%.

CONCLUSION

Full feeding during the rearing phase resulted in reduced pubic bones, ova, and oviducts development, delayed oviposition, and 20% egg production. Full feeding in the laying phase led to a higher laying percentage and egg weights despite the fully fed or restrictedly fed diets of chickens during the rearing phase. Therefore, Koekoek chickens should only be fed restrictedly during the rearing phase since their performance in terms of laying percentage, egg weight, and early peak egg production did not differ from those that were fully fed throughout the study. In the case where a farmer is interested in hatching, a feed restriction would be an ideal practice in order to maximize egg hatchability.

DECLARATIONS

Acknowledgements

The authors would like to thank the Department of Animal Science of the National University of Lesotho for providing facilities used in the current study.

Competing interests

The authors declare that they have no conflict of interest.

Author's contribution

Setsumi M. Molapo and Edward Webb developed the concept, analysed data and wrote the manuscript. Motselisi A. Mahlehla and Paseka Kompi assisted in data collection while Thato Chabeli designed the graphical abstract. All authors reviewed and confirmed the manuscript before submission.

REFERENCES

Adegbenro M, Ajidara SA, Modupe G, and Onibi EG (2020). Performance and Egg Qualities of Isa-Brown Layers Fed Different Quantities of Feed at Varying Feeding Frequencies. Turkish Journal of Agriculture - Food Science and Technology, 8(4): 864-872. DOI: https://doi.org/10.24925/turjaf.v8i4.864-872.3014

Cassy S, Metayer S, Crochet S, Rideau N, Collin A, and Tesserand S (2004). Leptin receptor in the chicken ovary: Potential involvement in ovarian dysfunction of ad libitum-fed broiler breeder hens. Reproductive Biology and Endocrinology, 2(72): 1- 8. DOI: https://doi.org/10.1186/1477-7827-2-72

Crounch AN, Grimes JL, Christensen VL, and Kruegert KK (2002). Effect of physical feed restriction during rearing on large white turkey breeder hens: 2. Reproductive performance. Poultry Science, 81: 16-22. DOI: https://doi.org/10.1093/ps/81.1.16

Evrim DF, and Kaya M (2014). Effects of early feed restriction on some performance and reproductive parameters in japanese quail). International Journal of Poultry Science, 13(6): 323-328. DOI: https://doi.org/ 10.3923/ijps.2014.323.328

Ezieshi VE, Nworu MJ, Bandele FO, Suleman RO, Ojurongbe BC, and Olomu JM (2003). Laying hen productivity in the tropics as affected by stage of egg production, feed restriction, stocking density and time of day. Archivos de Zootecnia, 52(200): 475-482. Available at: http://www.redalyc.org/articulo.oa?id=49520006

Melnychuk VL, Kirby JD, Kirby YK, Emmerson DA, and Anthony NB (2004). Effect of strain, feed allocation program, and age at photo stimulation on reproductive development and carcass characteristics of broiler breeder hens. Poultry Science, 83(11): 1861–1867. DOI: https://doi.org/10.1093/ps/83.11.1861

Miles RD, and Jacqueline PJ (2000). Feeding the commercial egg-type laying hen. factsheet ps049" department of dairy and poultry science. Florida cooperative extension service. University of Florida, Pp. 1-4. Available at: http://edis.ifas.ufl.edu/

Moreira RF, Freitas ER, Sucupira FS, Diógenes ALF, Abe MS, and Araújo FWS (2012). Effect of feed restriction with voluntary hay intake on the performance and quality of laying hen eggs. Acta Scientiarum Animal Sciences, 34(2): 149-154.
DOI: https://doi.org/10.4025/actascianimsci.v34i2.12451

Onagbesan OM, Metayer S, Tona K, Williams J, Decuypere E, and Bruggeman V (2006). Effects of genotype and feed allowance on plasma luteinizing hormones, Follicle Stimulating hormones, progesterones, estradiol levels, follicle differentiation, and egg production rates of broiler breeder hens. Poultry Science, 85(7): 1245-1258. DOI: https://doi.org/10.1093/ps/85.7.1245

Oyedeji JO, Orheruata AM, and Omatsuli M (2007). Effects of feed rationing on the laying performance of 40 weeks in lay hens. Journal of Food, Agriculture and Environment, 5: 301-303. DOI: https://doi.org/10.1234/4.2007.1205

Renema RA, Robinson FE, and Zuidhof MJ (2007). Reproductive efficiency and metabolism of female broiler breeders as affected by genotype, feed allocation, and age at photo stimulation. 2. Sexual maturation. Poultry Science, 86(10): 2267-2277. DOI: https://doi.org/10.1093/ps/86.10.2267

Renema RA, Robinson FE, Proudman JA, Newcombe M, and Mckay RA (1999). Effect of body weight and feed allocation during sexual maturation in broiler breeder. 2. Ovarian morphology plasma hormone profiles. Poultry Science, 78(5): 629–639. DOI: https://doi.org/10.1093/ps/78.5.629

Robinson FE, Zuidhof MJ, and Renema RA (2007). The reproductive efficiency and metabolism of female broiler breeders as affected by genotype feed allocation and age at photo stimulation. 1. Pullet growth and developments. Poultry Science, 86(10):2256-2266. DOI: https://doi.org/10.1093/ps/86.10.2256

Salih R , Tesfaye E , Tamir B, and Singh H (2016). Effects of Feed Restriction on Production Performance and Carcass Characteristics of Koekoek Chickens in Ethiopia. Poultry science Journal, 4 (1): 55-61. Available at: http://psj.gau.ac.ir

Simeneh G (2019). Review on the effect of feed and feeding on chicken performance. Animal Husbandry Dairy and Veterinary Science, 3: 1-4. DOI: https://doi.org/10.15761/AHDVS.1000171

Tesfaye E, Tamir B, Haile A, and Dessie T (2009). Effects of feed restriction on production and reproductive performance of Rhode island red pullets, 4(7). Available at: http://www.academicjournals.org/AJAR

Yildiz H, Yilmaz B, Arican I, Petek M, and Bahadir A (2006). Effects of cage systems and feeding time on the morphological structure of female genital organs in Pharaoh Quails (Coturnix coturnix pharaoh). Veterinarski Archive, 76(5): 381-389. Available at: https://hrcak.srce.hr/file/8254

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 571-579, December 25, 2020

Review Paper, PII: S2322455X2000065-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.65

Phytobiotics in Poultry Industry as Growth Promoters, Antimicrobials and Immunomodulators – A Review

Wafaa A. Abd El-Ghany

[1]*Poultry Diseases Department, Faculty of Veterinary Medicine, Cairo University, 1211, Giza, Egypt*

Corresponding author's E-mail: wafaa.ghany@yahoo.com; ORCID: 0000-0003-1686-3831

Received: 02 Nov. 2020
Accepted: 20 Dec. 2020

ABSTRACT

Due to the hazardous use of antimicrobials in poultry production sector, development of drug resistance have become a worldwide problem. Therefore, using biotic or natural products, such as phytobiotics (phytogenics or botanicals) have received a great attention as antibiotic substitutes. The use of phytobiotics or their constituents have been considered as a relatively new class of natural herbs that gained popularity and acceptability among poultry farmers. The incorporation of several types of phytobiotic additives in poultry feed have proved their ability to enhance the productive performance of broilers as well as layers. Moreover, phytbiotics presented great efficacy in counteracting intestinal pathogenic microorganism while maintaining the population of normal inhabitant beneficial microflora. Immunostimulatory effect on both humoral and cellular immunity as well as antioxidant properties were recorded as characters of phytobiotics. Therefore, this review article aimed to give a spotlight on the uses of different types of phytobiotics as poultry dietary additives to improve the productive parameters, reduce the pathogenic intestinal bacteria, and potentiate the immune response, especially after vaccination processes.

Keywords: Antimicrobial, Immunity, Performance, Plants, Poultry

INTRODUCTION

From several decades till now, poultry industry has been recognized as an important subsector of agricultural and veterinary fields due to increasing demand for meat and eggs as low cost protein sources. Continues feeding by sub-therapeutic levels of antimicrobials as growth promoter agents or antimicrobial compounds have had a negative impact on the balance of normal inhabitants of gut microflora, accumulation of antibiotic tissues residues as well as developing new strains of drug-resistant pathogenic bacteria (Castanon, 2007). Therefore, in 2006, the European Union Commission banned using of antibiotics in animal feeds as a growth promoter in different countries (Europe Union Commission, 2005).

Phytobiotics are also termed as phytogenics or botanicals. They are defined as natural, less toxic, and residue free plant-derived compounds that have been used as feed additives for livestock production (Wang et al., 2008). Phytobiotics are composed of natural bioactive components or substances of plant origin including terpenoids, alkaloids, glycosides and phenolics (Shad et al., 2014). Phytobiotics could be classified as herbs from flowering, non-woody and non-persistent plants, botanicals or spices from non-leaf parts like seeds, fruits, bark or root, essential oils or extracts and oleoresins (Bote, 2004).

Numerous studies have been conducted to demonstrate the effect of phytobiotics as growth promoter feed additives like prebiotics and probiotics to enhance overall performance parameters as well as health conditions of poultry (Yasodha et al., 2019; Özbudak, 2019). Phytobiotics have been also used as antimicrobial, antiparasitic, anticoccidial as well as immunostimulant agents in poultry field (Manafi, 2015; Gilani et al., 2018; Hafeez et al., 2020). Phytogenic substances were extremely studied in different species of monogastrics (Gheisar and Kim, 2018), rabbits (Alagawany et al., 2018; Al-Sagheer et al., 2019) and fish (Naiel et al., 2019).

So, the purpose of current article review was to spotlight on phytogenic compounds that used in poultry field and their effects on the productive performance, antimicrobial activities and immuno-stimulatory properties.

Production parameters

Improved growth parameters were detected in birds fed on different kinds of herbs, polysaccharides or essential oils components (Yasodha et al., 2019). The enhancement of the growth performance parameters after supplementation of phytobiotics may be depends on the synergistic mechanism among their active molecular complex (Hussein et al., 2020a). Phytobiotics could maintain or improve normal intestinal architecture, increase the villus length and consequently increase the surface of intestinal absorption (Tabatabaei, 2016). It has been demonstrated that phytobiotics are able to stimulate saliva production, secretion of digestible enzymes and bile production resulting in improving the performance and digestibility (Alloui et al., 2014). Moreover, phytobiotics enhance the digestion and digestion and utilization of protein in the intestine (El-Gendi, 1996), decrease the gut pathogens (Kubkomawa et al., 2013) and increase *Lactobacillus* spp. count (Windisch et al., 2008). It has been observed that supplementation with herbal feed additives can alter the histological structure of the intestine and indicated elevation of the intestinal villi by deepening of its crypts (Murali et al., 2012), increasing the dendritic cells absorption capacity in the intestinal lumen, stimulation of toll-like receptors and activation of epithelial to release the mucosal cytokines. Alcicek et al. (2004) assumed that feeding of broilers on phytobiotics stimulated the secretion of high amount of intestinal mucus and consequently reducing the pathogens adhesion and establishment of gut microbial eubiosis.

Supplementing broiler feed or water with essential oil mixtures of thymol and cinnamaldehyde (Tiihonen et al., 2010), thymol and star anise (Kim et al., 2016), clove and cinnamaldehyde (Chalghoumi et al., 2013), coriander (Ghazanfari et al., 2015; Hady et al., 2016), oregano (Hashemipour et al., 2014), a mixture of oregano, anise, and citrus peel (Abdelnaser et al., 2019), carvacrol (Jamroz et al., 2006), a blend of carvacrol, cinnamaldehyde and capsicum oleoresin (Bravo and Ionescu, 2008) and ginger extract (Olaifa et al., 2019) had been detected for improving all performance parameters including; feed intake, feed conversion rate and body weight. Moreover, supplementation of broilers diet with garlic (Elagib et al., 2013), a mixture of garlic, mushroom and propolis (Daneshmand et al., 2012), turmeric powder (Ahmadi, 2010), guggul resin (Iranparast et al., 2014), dried ground leaves of stevia (Atteh et al., 2008) and black cumin seeds (Khalaji et al., 2011) presented the enhancement of broilers performance. Studies on the effects of herbal compounds on the production of broilers Japanese quail's revealed improvement of all performances (Manafi et al., 2016).

The laying hens represented improvement in egg production, eggshell strength and thickness as well as internal egg quality after treatment with mixture of plant extracts and essential oils (Bölükbaşi et al., 2008; Radwan et al., 2008; Kaya et al., 2013).

Antimicrobial effect

It has been demonstrated that phytochemical compounds of phytobiotics have a strong antimicrobial activity against Gram-positive and Gram-negative bacteria either *in vivo* (Al-Kassie, 2010; Daka, 2013) or *in vitro* environment (Al-Mariri and Safi, 2014). Some phytochemical compounds as alkaloid inhibit DNA synthesis (Karou et al., 2005) and form saponin complexes with the cell membrane (sterols) which leading to cells damage and collapse (Morrissey and Osbourn, 1999). The antimicrobial effect of essential oils could be refer their ability to penetrate through the bacterial membrane (Helander et al., 1998), their chemical structure (Farag et al., 1989) and their aromaticity (Bowles and Miller, 1993).

Modulation of the gut microflora by phytogenic compounds plays an important role in maintaining host health (Tollba et al., 2012). Several studies revealed that phytogenic compounds or their extracts reduce the population of intestinal pathogenic organisms and their metabolites, but increase the number of intestinal normal and helpful microflora which relief the intestinal challenge and immune stress and consequently increase intestinal performance (Liu et al., 2014). It has been speculated that organic acids of some phytobiotic feed additives may lower the intestinal pH that leaded to inhibiting the pathogenicity of local pathogens and lowering the level of their toxic products (Manafi et al., 2016).

Antibacterial effect

Essential oils could reduce the growth of common intestinal poultry pathogens. Inoculation of thyme and cinnamon in the broiler ration reduces the total bacterial as well as coliform count in the intestinal tract (Karangiya et al., 2016). An *in vitro* study revealed that flowers of Calendula presented growth inhibitory effects on *Escherichia coli* (*E. coli*) (Arora et al., 2013). However, some reports indicated the inhibitory effects of dietary phytobiotics or plant extracts on *E. coli* activity *in vivo* (Abd El-Ghany and Ismail, 2014; Diaz-Sanchez et al., 2015; Elmenawey et al., 2019). Diet containing thyme essential oil helped for increasing in *Lactobacillus* and decreasing in *E. coli* counts in the intestine of Japanese

quail (Khaksar et al., 2012). Essential oils of oregano revealed antimicrobial properties on broiler carcasses through reduction of the total bacterial count especially *Salmonella* spp. (Aksit et al., 2006). Short chain fatty acids of phytobiotics revealed growth promoting effect on the intestinal beneficial microbiota as well as controlling the growth of *Salmonella enteritidis* (*S. enteritids*) (Hansen et al., 1997). Quail's diet containing phytobiotic feed additives had significant (P≤0.05) increase in the number of *Lactobacilli*, decrease in *E. coli* population and inhibit the growth of *Salmonella* in the intestinal tract (Dorman and Deans, 2000).

The efficacy of a mixture of seven essential oils that inhibited the growth of *Clostridium perfringens* (*C. perfringens*) *in vitro* has been reported by Si et al. (2009). In the field studies of Mitsch et al. (2004), Siragusa et al. (2008), McReynolds et al. (2009), Abudabos et al. (2018), El-Sheikh et al. (2018) and Hussein et al. (2020b), different phytobiotic feed additives caused reduction of intestinal colonization and proliferation of *C. perfringens*, lesion score and mortalities as well as improvement in performance of broilers and their carcass quality.

The reduction in *C. perfringens* virulence after supplementation with essential oil compounds may be related to the stimulation of some digestible enzymes like trypsin which inactivates α toxin of type A and β toxin of type C strains of *C. perfringens*, stabilization of natural resident gut microflora like *Lactobacillus* spp. and consequently inhibition of the organism pathogenicity (Cho et al., 2014).

Moreover, essential oils of lemon, green tea and turmeric blend proved great efficacy in reducing the count of *S. enteritidis* and *Campylobacter jejuni* on the surface of chicken's carcass (Murali et al., 2012). The Eucalyptus volatile oils have been found to have the ability to relief broilers complicated respiratory distress caused by Mycoplasma gallisepticum (Abd El-Ghany, 2008).

Antifungal and detoxifying effect

Phytogenic compound have antifungal properties. Akgul and Kivanc (1988) reported about the inhibitory effects of some spices and oregano components on some foodborne fungi. Essential oil of marjoram reduced the *in vitro* growth of *Aspergillus flavus*, *A. niger*, *A. ochraceus* and *A. parasiticus* up to 89% (Deans and Waterman, 1993). The oil of lemon and orange can reduce the formation of *A. flavus* (Hasan et al., 2005). It has been demonstrated that *Allium sativum* has a great (60-80%) *in vitro* antifungal activity against *Aspergillus* and *Penicillium* spp. (Afzal et al., 2010). Onion, garlic and ginger revealed antifungal activity against *A. flavus*, *A. niger* and *Cladosporium herbaru*, *Cinnamomum verum* and *Piper nigrum* which was studied with successful results by Tagoe et al. (2011). Phytobiotics *like Ocimum gratissimum, Cymbopogon citratus, Xylopia aethiopica, Monodora myristica*, *Syzygium aromaticum* proved their ability to inhibit the formation of non sorbic acid which is a precursor in the pathway of aflatoxin synthesis (Awuah, 1996). Neem extract represented inhibitory effects on biosynthesis of aflatoxins (B and G) (Bhatnagar et al., 1990). Also, neem leaf extract and its oil have been found to inhibit the growth and morphology of *Penicillium* spp. and consequently prevent the production of ochratoxin A (Bhatnagar et al., 1990).

Anticoccidial effect

The anticoccidial activity of some herbal plants have been documented (Willis et al., 2013). Phytogenic compounds have been found to reduce the severity of *Eimeria* spp. infection in broilers by alleviation of droppings score, intestinal lesions score and also reducing oocyst shedding (Zyan et al., 2017). Numerous phytobiotics as Atemesia annua (Allen et al., 1997), *Astragulus membranaceus* and *Sophora flavescens* (Youn and Noh, 2001), green tea (Jang et al., 2007), *Ageratum conyzoid* (Nweze and Obiwulu, 2009), *Musa paradisiaca* (Anosa and Okoro, 2010), olive tree (De Pablos et al., 2010), oregano essential oil (Tsinas et al., 2011), *Carica papaya* leaf extract (Nghonjuyi et al., 2015) and coconut oil (Hafeez et al., 2020) have indicated an excellent anticoccidial activity against different types of *Eimeria* spp. in birds.

Some essential oils presented similar drug efficacy in prevention and control of coccidiosis in broilers. The oregano oil and other mixtures of oils were similar to ionophores lasalocid (Giannenas et al., 2003) and monensin (Oviedo-Rondón et al., 2006), while a mixture of carvacrol, camphor, cineole and thymol was similar to salinomycin (Bozkurt et al., 2014) in terms of reduction of shedding and lesions of different *Eimeria* spp. in broilers.

Combined experimental infection of *C. perfringens* and *E. maxima* has been ameliorated after dietary treatment of three breeds of broilers with Capsicum, Curcuma longa oleoresins (Kim et al., 2015) and Allium hookeri root (Lee et al., 2018).

Immunomodulatory effect

In poultry production, reduction of infection as well as improvement of production by stimulation of the immune system after using phytogenic substances were

investigated previously (Zaki et al., 2016). The immunomodulatory mechanism of phytogenic active substances in poultry have been studied (Hashemi and Davoodi, 2012). Polysaccharide are very important immunoactive components of phytobiotics (Xue and Meng, 1996). Phytogenic compounds also induce their immunomodulatory effects through increasing immune cells proliferation, arising cytokines expression and elevation of antibody titers (Lee et al., 2010; Park et al., 2011; Pourhossein et al., 2015). The immunogenicity of phytobiotics could be manifested as increasing macrophages, lymphocytes and natural killer cells activities as well as stimulation of interferon production (Hashemi and Davoodi, 2010; Kumar et al., 2014). Plants contain flavonoids, vitamin C and carotenoids are able to enhance the immune system (Craig, 1999). Some herbs and species like garlic, echinacea and liquoric have immunostimulatory properties due to their composition of vitamin C, carotenoids and flavonoids as well as their abilities to stimulate macrophages, lymphocytes and natural killer cells activities and interferon production (Frankic et al., 2009). Detecting *in vitro* immuno-stimulatory effect of dandelion, mustard and safflower either on lymphocytes and macrophages of chickens was performed by Lee et al. 2007. The results indicated inhibition of tumor cell growth, antioxidant effects, stimulation of lymphocyte proliferation and nitric oxide production by macrophages. The immunostimulant effect of some essential oils extracts of phytobiotics may be due to the presence of certain compounds that may bind to Immunoglobulin G (Ig G) receptors which leaded to stimulation of immune response (Ahmed et al., 2013). In the study of Placha et al. (2014), inoculation of thymol in the broiler diet increased the trans-epithelial electrical resistance of duodenal mucosa.

It has been found that mushroom and plant polysaccharides have immunomodulatory effects in chickens infested with *Eimeria tenella* (Guo et al., 2004).

Potential antiviral activity of plant seeds was recorded (Yaseen, 2003). Different types of herbs mix, spices, plant extracts and essential oils presented enhancement of immune response of birds (Huang et al., 2007; Pourali et al., 2010; Kavyani et al., 2012; Abou-Elkhair et al., 2014; Awaad et al., 2016).

A significant ($P<0.05$) elevation of antibody Enzyme-linked immunosorbent assay (ELISA) titer after vaccination with Newcastle Disease Virus (NDV) vaccine (Chowdhury et al., 2018) was recorded after feeding on clove bud and cinnamon plant, while peppermint essential oil helped in significant ($P<0.05$) rising in Haemagglutination Inhibition (HI) antibody titer against Avian Influenza (AI) virus vaccines (H9N2) in broiler chicks (Sultan et al., 2017). In addition, significant ($P<0.05$) increase in HI titers against NDV vaccine and sheep red blood cells were observed in broiler chicks treated with Aloe vera gel (1%) in the drinking water for six weeks (Darabighane et al., 2017)

Laying hens presented significant ($P>0.05$) increase in ELISA titer after vaccination with ND, Infectious Bronchitis (IB) and Infectious Bursal Disease (IBD) vaccines (Özek et al., 2011). Landy et al. (2011) observed that inoculation of broiler ration with neem powder for six weeks resulting in an elevation of HI antibody titer against AI vaccine, but not against ND vaccine. Barbour et al. (2008) evaluated the effect of using Eucalyptus and peppermint essential oils during vaccination of ND and IBD. The results indicated improved health conditions of the birds associated with elevation of ELISA titers against the used virus vaccines. Moreover, an increase in IgG and IgM ELISA titers has been observed in chickens fed on oregano essential oils for three weeks period (Malayoglu et al., 2010). Recently, Abdelnaser et al. (2019) indicated that treatment with essential oils of oregano, anise, and citrus peel at level of 125 gm/ton induced positive effects on the immune response of *C. perfringens* challenged broilers after vaccination against ND, IB, AI and IBD viruses as well as increasing in relative spleen weight.

CONCLUSION

Using of phytobiotics in poultry diet as a feed additive and considering them as an antimicrobial substitute has been became a very essential and critical issue currently. Phytobiotic compounds could replace antibiotic growth promoters to improve the productive performance of chickens, act as antibacterial, antifungal and antiparasitic agents as well as it has been considered as potential immunostimulants especially after routine vaccination programs of chickens. This review spotlight on the significant using of phytobiotics in poultry field and industry as natural antibiotic alternatives to avoid the emerged problem of antibiotic resistance,

Competing interests

The author have not declared any conflict of interest.

REFERENCES

Abd El-Ghany WA (2008). Assessment of the efficacy of certain antibiotics and volatile oils for the treatment of induced ycoplasma gallisepticum infection in broiler chickens. Journal of the Egyptian

Veterinary Medical Association, 69: 283-307. Avaialble at: https://scholar.cu.edu.eg/?q=wafaaabdelghany/publications/assessment-efficacy-certain-antibiotics-and-volatile-oils-treatment-ind

Abd El-Ghany WA and Ismail M (2014). Tackling experimental colisepticaemia in broiler chickens using phytobiotic essential oils and antibiotic alone or in combination. A mixture of essential oils of *Oreganum aetheroleum* is more effective compared with ciprofloxacin in the treatment of *E. coli* in broiler chickens. Iranian Journal of Veterinary Research, 15: 110-115. DOI: https://www.dx.doi.org/10.22099/ijvr.2014.2341

Abdelnaser AHT, Moustafa MMA and Abd El-Haleem MI (2019). Effect of using synbiotics and essential oils on performance parameters and immune response of necrotic enteritis challenged broiler chicks. Benha Veterinary Medical Journal, 36: 373-380. DOI: https://www.dx.doi.org/10.21608/BVMJ.2019.15094.1051

Abudabos AM, Alyemni AH, Dafalla YM and Khan RU (2018). The effect of phytogenics on growth traits, blood biochemical and intestinal histology in broiler chickens exposed to *Clostridium perfringens* challenge. Journal of Applied Animal Research, 46: 691-695. DOI: https://www.doi.org/10.1080/09712119.2017.1383258

Abou-Elkhair R, Ahmed HA and Selim S (2014). Effects of black pepper (*Piper nigrum*), turmeric powder (*Curcuma longa*) and coriander seeds (*Coriandrum sativum*) and their combinations as feed additives on growth performance, carcass traits, some blood parameters and humoral immune response of broiler chickens. Asian Australasian Journal of Animal Sciences, 27: 847-854. DOI:https://www.dx.doi.org/10.5713%2Fajas.2013.13644

Afzal R, Mughal S M, Munir M, Sultana K, Qureshi R, Arshad M and Laghari MK (2010). Mycoflora associated with seeds of different sunflower cultivars and its management. Pakistan Journal of Botany, 42: 435-345. Available at: http://www.pakbs.org/pjbot/PDFs/42(1)/PJB42(1)435.pdf

Ahmed ST, Hossain ME, Kim GM, Hwang JA, Ji H and Yang CJ (2013). Effects of resveratrol and essential oils on growth performance immunity, digestibility and fecal microbial shedding in challenged piglets. Asian Australasian Journal of Animal Sciences, 26: 683-690. DOI: https://www.dx.doi.org/10.5713%2Fajas.2012.12683

Ahmadi F (2010). Effect of turmeric (*Curcumin longa*) powder on performance, oxidative stress state and some of blood parameters in broilers fed on diets containing aflatoxin. Global Veterineria, 5: 312-317. Available at: http://www.idosi.org/gv/gv5(6)10/4.pdf

Akgul A, and Kivanc M (1988). Inhibitory effects of selected Turkish spices and oregano components on some foodborne fungi. . International Journal of Food Microbiology, 6: 263-268. DOI: https://www.doi.org/10.1016/0168-1605(88)90019-0

Aksit M, Goksoy E, Kok F, Ozdemir D and Ozdogan M (2006). The impacts of organic acid and essential oil supplementations to diets on the microbiological quality of chicken carcasses. Archiv fur Geflugelkund, 70: 168-173. Available at: https://www.european-poultry-science.com/The-impacts-of-organic-acid-and-essential-oil-supplementations-to-133diets-on-the-microbiological-quality-of-chickencarcasses,QUlEPTQyMTcxOTQmTUlEPTE2MTAxNA.html

Alagawany M, AbdEl-Hack ME, Al-Sagheer AA, Naiel MA, Saadeldin IM and Swelum AA (2018). Dietary cold pressed water cress and coconut oil mixture enhances growth performance, intestinal microbiota, antioxidant status, and immunity of growing rabbits. Animals, 8: 212. DOI: https://www.dx.doi.org/10.3390%2Fani8110212

Alcicek A, Bozkurt M and Çabuk M (2004). The effect of a mixture of herbal essential oils, an organic acid or a probiotic on broiler performance. South African Journal of Animal Science 34: 217-222. Available at: https://hdl.handle.net/10520/EJC94382

Al-Kassie GAM (2010). The effect of thyme and cinnamon on the microbial balance in gastro intestinal tract on broiler chicks. International Journal of Poultry Science, 9: 495-498. DOI: http://www.dx.doi.org/10.3923/ijps.2010.495.498

Allen PC, Lydon J and Danforth H (1997). Effects of components of *Artemisia annua* on coccidia infections in chickens. Poultry Science, 76: 1156-1163. DOI: https://www.doi.org/10.1093/ps/76.8.1156

Alloui N, Alloui MN, Agabou A (2014). Application of herbs and phytogenic feed additives in poultry production - a review. Global Journal Animal Science Research, 2: 234-243. Available at:http://archives.gjasr.com/index.php/GJASR/article/view/57

Al-Mariri A and Safi M (2014). *In vitro* antibacterial activity of several plant extracts and oils against some gram-negative bacteria. Iranian Journal of Medical Sciences, 39: 36. Available at:https://www.ncbi.nlm.nih.gov/pubmed/24453392

Al-Sagheer AA, AbdEl-Hack ME, Alagawany M, Naiel MA, Mahgoub SA, Badr MM, Hussein EO, Alowaimer AN and Swelum AA (2019). Paulownia leaves as a new feed resource: Chemical composition and effects on growth, carcasses, digestibility, blood biochemistry, and intestinal bacterial populations of growing rabbits. Animals, 9: 95. DOI: https://www.dx.doi.org/10.3390%2Fani9030095

Anosa GN and Okoro OJ (2010). Anticoccidial activity of the methanolic extract of *Musa Paradisiaca* root in chickens. Tropical Animal Health and Production, 43: 245-248. DOI: http://www.doi.org/10.1007/s11250-010-9684-1

Arora D, Rani A and Sharma A (2013). A review on phytochemistry and ethnopharmacological aspects of genus *Calendula*. Pharmacognosy Reviews, 7: 179-187. Available at: https://www.ncbi.nlm.nih.gov/pubmed/24347926

Atteh JO, Onagbesan OM, Tona K, Decuypere E, Geuns JM and Buyse J (2008). Evaluation of supplementary stevia (*Stevia rebaudiana, bertoni*) leaves and stevioside in broiler diets: effects on feed intake, nutrient metabolism, blood parameters and growth performance. Journal of Animal Physiology and Animal Nutrition, 92: 640-649. DOI: https://www.doi.org/10.1111/j.1439-0396.2007.00760.x

Awaad MHH, Afify MAA, Zoulfekar SA, Mohammed FF, Elmenawy MA and Hafez HM (2016). Modulating effect of peppermint and eucalyptus essential oils on vVND infected chickens. Pakistan Veterinary Journal, 36: 350-355. Available at: http://www.pvj.com.pk/pdf-files/36_3/350-355.pdf

Awuah RT (1996). Possible utilization of plant products in grain storage. Proceedings of the Workshop on Mycotoxins in Food in Africa, Nov. 6-10, International Institute of Tropical Agriculture, Benin, p. 32. Available at: http://www.academicjournals.org/AJB

Barbour EK, Yaghi RH, Shaib HA, Tayeb IT and Sleiman FT (2008). Evaluation of an essential oil in treatment of immunosuppressed coinfected broilers. Eurasian Journal of Sustainable Agriculture, 2: 212-218. Available at: http://www.aensi.org/aejsa/2008/212-218.pdf

Bhatnagar D, Zeringue HJ and McCormick SP (1990). Neem leaf extracts inhibit aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus*. Proceedings of the USDA Neem Workshop, April 16-17, Beltsville, Maryland, pp. 118-127. Avaialble at: https://agris.fao.org/agris-search/search.do?recordID=US9164967

Bölükbaşi ŞC, Erhan MK and Kaynar Ö (2008). The effect of feeding thyme, sage and rosemary oil on laying hen performance, cholesterol and some proteins ratio of egg yolk and *Escherichia coli* count in feces. Archiv fur Geflugelkund, 72: 231-237. Available at: https://www.european-poultry-science.com/The-effect-of-feeding-thyme-sage-and-rosemary-oil-on-laying-hen-performance-cholesterol-and-some-proteins-ratio-of-egg-yolk-and-span-classws-name-Escherichia-Colispan-cou,QUlE

Bote CJL (2004). Bioflavonoid's effects reach beyond productivity. Feed Mix, 12: 12-15.

Bowles BL and Miller AJ (1993). Antibotulinal properties of selected aromatic and aliphatic aldehydes. Journal of Food production, 56: 788-794. DOI: https://www.doi.org/10.4315/0362-028x-56.9.788

Bozkurt M, Aysul N, Küçükyilmaz K, Aypak S, Ege G, Çatli A U, Akúit H, Çöven F, Seyrek K and Çınar M (2014). Efficacy of in-feed preparations of an anticoccidial, multienzyme, prebiotic, probiotic, and herbal essential oil mixture in healthy and *Eimeria* spp.-infected broilers. Poultry Science, 93: 389-399. DOI: https://www.doi.org/10.3382/ps.2013-03368

Bravo D, and Ionescu C (2008). Meta-analysis of the effect of a mixture of carvacrol, cinnamaldehyde and capsicum oleoresin in broilers. Poultry Science, 87: 75. Available at: https://www.researchgate.net/publication/298327084_Metaanalysis_of_the_effect_of_a_mixture_of_carvacrol_cinnamaldehyde_and_capsicum_oleoresin_in_broilers

Castanon JIR (2007). History of the use of antibiotic as growth promoters. Journal of Poultry Science, 86: 2466-2471. DOI: https://www.doi.org/10.3382/ps.2007-00249

Chalghoumi R, Belgacem A, Trabelsi I, Bouatour Y and Bergaoui R (2013). Effect of dietary supplementation with probiotic or essential oils on growth performance of broiler chickens. International Journal of Poultry Science, 12: 538-344. DOI: http://www.dx.doi.org/10.3923/ijps.2013.538.544

Cho JH, Kim HJ and Kim IH (2014). Effects of phytogenic feed additive on growth performance, digestibility, blood metabolites, intestinal microbiota, meat color and relative organ weight after oral challenge with *Clostridium perfringens* in broilers. Livestock Science, 160: 82-88. DOI: https://www.doi.org/10.1016/j.livsci.2013.11.006

Chowdhury S, Mandala G, Patraa A, Kumara P, Samantab I, Pradhanc S, Chowdhurya A, Mandala G, Patraa A, Kumara P et al. (2018). Different essential oils in diets of broiler chickens: 2. Gut microbes and morphology, immune response, and some blood profile and antioxidant enzymes. Animal Feed Science and Technology, 236: 39-47. DOI: https://www.doi.org/10.1016/j.anifeedsci.2017.12.003

Craig WJ (1999). Health-promoting properties of common herbs. American Journal of Clinical Nutrition, 70: 491-499. DOI: https://www.doi.org/10.1093/ajcn/70.3.491s

Daka D (2013). Antibacterial effect of garlic (*Allium sativum*) on *Staphyloccus aureus*: An *in vitro* study. African Journal of Biotechnology, 10: 666-669. Available at: http://www.academicjournals.org/AJB

Daneshmand A, Sadeghi GH and Karimi A (2012). The effects of a combination of garlic, oyster mushroom and propolis extract in comparison to antibiotic on growth performance, some blood parameters and nutrients digestibility of male broilers. Brazilian Journal of Poultry Science, 14: 141-147. DOI: https://www.doi.org/10.1590/S1516-635X2012000200009

Darabighane B, Mahdavi A, Aghjehgheshlagh F, Zarei A, Kasapidou El and Nahashon S (2017). Effect of Aloe vera and vitamin E supplementation on the immune response of broilers. Revista Colombiana de Ciencias Pecuarias, 30: 159-164. DOI: http://www.dx.doi.org/10.17533/udea.rccp.v30n2a07

Deans SG and Waterman PG (1993). Biological Activities of Volatile Oils. In: Volatile Oil Crops: Their Biology, Biochemistry and Production, Hay, R.K.M. and P.G. Waterman (Eds.). Longman, London, UK, pp. 97-111. Available at: https://agris.fao.org/agris-search/search.do?recordID=GB9405744

De Pablos LM, dos Santos MF, Montero E, Garcia-Granados A, Parra A and Osuna A (2010). Anticoccidial activity of maslinic acid against infection with *Eimeria tenella* in chickens. Parasitology Research, 107: 601-604. DOI: https://www.doi.org/10.1007/s00436-010-1901-3

Diaz-Sanchez S, D'Souza D, Biswas D and Hanning I (2015). Botanical alternatives to antibiotics for use in organic poultry production. Poultry Science, 94: 1419-1430. DOI: https://www.doi.org/10.3382/ps/pev014

Dorman HJ and Deans SG (2000). Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. Journal of Applied Microbiology, 88: 308-316. DOI: https://www.doi.org/10.1046/j.1365-2672.2000.00969.x

Elagib HAA, El-Amin WIA, Elamin KM and Malik HEE (2013). Effect of dietary garlic (*Allium sativum*) supplementation as feed additive on broiler performance and blood profile. Journal of Animal Science Advances, 3: 58-64. Available at: Link

El-Gendi GM (1996). Effect of feeding dietary herbal feed additives on productive and metabolic responses of broiler chicks. Egyptian Poultry Science, 16: 395-412. Available at: https://www.scirp.org/(S(czeh2tfqyw2orz553k1w0r45))/reference/ReferencesPapers.aspx?ReferenceID=1656208

Elmenawey MA, Mohammed FA, Morsy EA, Abdel-Alim GA and Awaad MHH (2019). The impact of essential oils blend on experimental colisepticemia in broiler chickens. International Journal of Veterinary Science, 8: 294-299. Available at: http://www.ijvets.com/pdf-files/Volume-8-no-4-2019/294-299.pdf

El-Sheikh SM, Khairy MH, Eleiwa NZ, Abdalla OE and El-Monsef AGA (2018). Effect of sanguinarine phytobiotic, sodium butyrate compared to ampicillin on controlling necrotic enteritis in broiler chickens. In Proceedings of the Veterinary Medicine In-between Health & Economy (VMHE), Cairo, Egypt, 16–19 October 2018,Volume 55. DOI: https://doi.org/10.26873/SVR-668-2018

Europe Union Commission (2005). Ban on antibiotics as growth promoters in animal feed enters into effect. Regulation 1831/2003/EC on additives for use in animal nutrition, replacing Directive 70/524/EEC on additives in feed- stuffs, Brussels, 22 December. Available at: https://ec.europa.eu/commission/presscorner/detail/en/IP_05_1687

Farag RS, Badei AZMA, Hewedi FM and El-Baroty GSA (1989). Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. Journal of the American Oil Chemists' Society, 66: 792-799. DOI: https://www.doi.org/10.1007/BF02653670

Frankic T, Voljg M, Salobir J and Rezar V (2009). Use of herbs and spices and their extracts in animal nutrition. Actaagriculturae Slovenica, 92: 95-102. Available at: http://aas.bf.uni-lj.si/zootehnika/94-2009/PDF/94-2009-2-95-102.pdf

Ghazanfari S, Mohammadi Z and Moradi AM (2015). Effects of coriander essential oil on the performance, blood characteristics, intestinal microbiota and histology of broilers. Brazilian Journal of Poultry Science, 17: 419-426. DOI: https://www.doi.org/10.1590/1516-635X1704419-426

Gheisar MM and Kim IH (2018). Phytobiotics in poultry and swine nutrition – a review. Italian Journal of Animal Science, 17: 92-99. DOI: https://www.doi.org/10.1080/1828051X.2017.1350120

Giannenas I, Florou-Paneri P, Papazahariadou M, Christaki E, Botsoglou NA and Spais AB (2003). Effect of dietary supplementation with oregano essential oil on performance of broilers after experimental infection with *Eimeria tenella*. Archives of Animal Nutrition, 57: 99-106. DOI: https://www.doi.org/10.1080/0003942031000107299

Gilani SMH, Zehra S, Hassan F, Galani S and Ashraf A (2018). Effect of natural growth promoters on immunity, and biochemical and haematological parameters of broiler chickens. Tropical Journal of Pharmaceutical Research, 17: 627-633. DOI: https://www.doi.org/10.4314/tjpr.v17i4.9

Guo FC, Kwakkel RP, Williams BA, Parmentier HK, Li WK, Yang ZQ and Verstegen MW (2004). Effects of mushroom and herb polysaccharides on cellular and humoral immune responses of *Eimeria tenella*-infected chickens. Poultry Science, 83: 1124-1132. DOI: https://www.doi.org/10.1093/ps/83.7.1124

Hady MM, M Zaki MM, Abd El-Ghany WA and Korany RMS (2016). Assessment of the broilers performance, gut healthiness and carcass characteristics in response to dietary inclusion of dried coriander, turmeric and thyme. International Journal of Environmental and

Agriculture Research, 2: 153-159. Available at: https://issuu.com/ijoear-environmentalandagriculturer

Hafeez A, Ullah Z, Khan RU, Qudrat U and Naz S (2020). Effect of diet supplemented with coconut essential oil on performance and villus histomorphology in broiler exposed to avian coccidiosis. Tropical Animal Health and Production, 52: 2499-2504. DOI: https://www.doi.org/10.1007/s11250-020-02279-6

Hansen LL, Larsen AE, Jensen BB and Hansen-Moller J (1997). Short time effect of zinc bacitracin and heavy fouling with faeces plus urine on boar taint. Journal of Animal Science, 64: 351-563. DOI: https://www.doi.org/10.1017/S1357729800015927

Hasan MM, Chowdhury SP, Alam S, Hossain B and Alam MS (2005). Antifungal effects of plant extracts on seed-borne fungi of wheat seed regarding seed germination, Seedling health and vigour index. Pakistan Journal of Biological Sciences, 8: 1284-1289. DOI: http://www.dx.doi.org/10.3923/pjbs.2005.1284.1289

Hashemi SR and Davoodi H (2010). Phytogenics as new class of feed additive in poultry industry. Journal of Animal and Veterinary Advances**,** 9: 2295-2304. DOI: http://www.dx.doi.org/10.3923/javaa.2010.2295.2304

Hashemi SR and Davoodi H (2012). Herbal plants as new immuno-stimulator in poultry industry: A review. Asian Journal of Animal and Veterinary Advances, 7: 105-116. DOI: http://www.dx.doi.org/10.3923/ajava.2012.105.116

Hashemipour H, Kermanshahi H, Golian A and Khaksar V (2014). Effects of carboxy methyl cellulose and thymol + carvacrol on performance, digesta viscosity and some blood metabolites of broilers. Journal of Animal Physiology and Animal Nutrition, 98: 672-679. DOI: https://www.doi.org/10.1111/jpn.12121

Helander IM, Alakomi HL, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid EJ, Gorris LGM and von Wright A (1998). Characterization of the action of selected essential oil components on gram-negative bacteria. Journal of Agricultural and Food Chemistry, 46: 3590-3595. DOI: https://www.doi.org/10.1021/jf980154m

Huang RL, Deng ZYC, Yang YL, Yin Xie MY, Wu GY, Li TJ, Li LL, Tang ZR, Kang P, Hou ZHP et al. (2007). Dietary oligochitosan supplementation enhances immune status of broilers. Journal of Science of Food and Agriculture, 87: 153-159. DOI: https://www.doi.org/10.1002/jsfa.2694

Hussein EOS, Ahmed SH, Abudabos AM, Suliman GM, Abd El-Hack ME, Swelum AA and Alowaimer AN (2020a). Ameliorative effects of antibiotic-, probiotic- and phytobiotic-supplemented diets on the performance, intestinal health, carcass traits, and meat quality of *Clostridium perfringens*-infected broilers. Animals 10: 669. DOI: https://www.doi.org/10.3390/ani10040669

Hussein EOS, Ahmed SH, Abudabos AM, Aljumaah MR, Alkhlulaifi MM, Nassan MA, Suliman GM, Naiel MAE and Swelum AA (2020b). Effect of antibiotic, phytobiotic and probiotic supplementation on growth, blood indices and intestine health in broiler chicks challenged with *Clostridium perfringens*. Animals, 10: 507. DOI: https://www.doi.org/10.3390/ani10030507

Iranparast F, Parsaei S, Houshmand M and Naghiha A (2014). The effect of oral consumption of guggul (*Commiphora mukul*) resin on performance and humoral immunity response of broilers. International Journal of Advanced Biological and Biomedical Research, 2: 802-810. Available at: http://www.ijabbr.com/article_7237_c88f2b6f14b9d63e28ab48d317528e06.pdf

Jamroz D, Wertelecki T, Houszka M and Kamel C (2006). Influence of diet type on the inclusion of plant origin active substances on morphological and histochemical characteristics of the stomach and jejunum walls in chicken. Journal of Animal Physiology and Animal Nutrition, 90: 255-268. DOI: https://www.doi.org/10.1111/j.1439-0396.2005.00603.x

Jang SI, Jun MH, Lillehoj HS, Dalloul RA, Kong Il-K, Kim S and Min W (2007). Anticoccidial effect of green tea-based diets against *Eimeria maxima*. Veterinary Parasitology, 144: 172-175. DOI: https://www.doi.org/10.1016/s0304-4017(01)00385-5

Karangiya VK, Savsani HH, Patil SS, Garg DD, Murthy KS, Ribadiya NK and Vekariya SJ (2016). Effect of dietary supplementation of garlic, ginger and their combination on feed intake, growth performance and economics in commercial broilers. Veterinary World, 9: 245-250. Availble at: https://www.ncbi.nlm.nih.gov/pubmed/27057106

Karou D, Savadogo A, Canini A, Yameogo S, Montesano C, Simpore J, Colizzi V and Traore A S (2005). Antibacterial activity of alkaloids from Sida acuta. African Journal of Biotechnology, 4: 1452-1457. Available at: http://www.academicjournals.org/AJB

Kavyani A, Zare Shahne A, PorReza J, Jalali Haji-abadi SMA and Landy N (2012). Evaluation of dried powder of mushroom (*Agaricus bisporus*) as an antibiotic growth promoter substitution on performance, carcass traits and humoral immune responses in broiler chickens. Journal of Medicinal Plants Research, 6: 94-100. DOI: https://www.doi.org/10.5897/JMPR11.1168

Kaya A, Kaya H, Macit M, Celebi S, Esenbuga N, Yoruk MA and Karaoglu M (2013). Effects of dietary inclusion of plant extract mixture and copper into layer diets on egg yield and quality, yolk cholesterol and fatty acid composition. Kafkas Universitesi Veteriner Fakultesi Dergisi, 19: 673-679. Available at:http://vetdergi.kafkas.edu.tr/extdocs/2013_4/673-679.pdf

Khaksar V, Van Krimpen MM, Hashemipour H and Pilevar M (2012). Effects of thyme essential oil on performance, some blood parameters and ileal microflora of Japanese quail. Journal of Poultry Science, 49: 106-110. DOI: https://www.doi.org/10.2141/jpsa.011089

Khalaji S, Zaghari M, Hatami K, Hedari-Dastjerdi S, Lotfi L and Nazarian H (2011). Black cumin seeds, Artemisia leaves (*Artemisia sieberi*), and *Camellia L*. plant extract as phytogenic products in broiler diets and their effects on performance, blood constituents, immunity, and cecal microbial population. Poultry Science, 90: 2500-2510. DOI: https://www.doi.org/10.3382/ps.2011-01393

Kim SJ, Lee KW, Kang CW and An BK (2016). Growth performance, relative meat and organ weights, cecal microflora, and blood characteristics in broiler chickens fed diets containing different nutrient density with or without essential oils. Asian Australasian Journal of Animal Science, 29: 549-554. DOI: https://www.dx.doi.org/10.5713%2Fajas.15.0426

Kim JE, Lillehoj HS, Hong YH, Kim GB, Lee SH, Lillehoj EP and Bravo DM (2015). Dietary Capsicum and *Curcuma longa* oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds. Research in Veterinary Science, 102: 150-158. DOI: https://www.doi.org/10.1016/j.rvsc.2015.07.022

Kubkomawa HI, Nafarnda DW, Mukang SM, Tizhe MA, Tuakam DK, Shua NJ, Ugwu CC, Opara MN, Neils JS and Okoli IC (2013). Ethno-Veterinary health management practices amongst livestock producers in Africa – A review. World Journal of Agricultural Sciences, 1: 252-257. Available at:http://wsrjournals.org/journal/wjas

Kumar M, Kumar V, Roy D, Kushwaha R and Vaiswani S (2014). Application of herbal feed additives in animal nutrition – a review. International Journal of Livestock Research 4: 1-8. DOI: https://www.dx.doi.org/10.5455/ijlr.20141205105218

Landy N, Ghalamkari G, Toghiani M and Yazdi F (2011). Humoral immune responses of broiler chickens fed with antibiotic and neem fruit powder (*Azadirachta Indica*) as feed additive supplemented diet. International Conference of Life Science and Technology (IPCBEE), 3: 153–155. Available at: http://www.ipcbee.com/vol3/39-L20019.pdf

Lee YS, Lee SH, Gadde UD, Oh ST, Lee SJ and Lillehoj HS (2018). *Allium hookeri* supplementation improves intestinal immune response against necrotic enteritis in young broiler chickens. Poultry Science, 97: 1899-1908. DOI: https://www.doi.org/10.3382/ps/pey031

Lee SH, Lillehoj HS, Chun HK, Tuo W, Park HJ, Cho SM, Lee YM and Lillehoj EP (2007). *In vitro* treatment of chicken peripheral blood lymphocytes, macrophages and tumor cells with extracts of Korean medicinal plants. Nutrition Research, 27: 362-366. DOI: https://www.doi.org/10.1016/j.nutres.2007.04.001

Lee SH, Lillehoj HS, Hong YH, Jang SI, Lillehoj EP, Ionescu C, Mazuranok L and Bravo D (2010). *In vitro* effects of plant and mushroom extracts on immunological function of chicken lymphocytes and macrophages. British Poultry Science, 51: 213-221. DOI: https://www.doi.org/10.1080/00071661003745844

Liu HN, Liu Y, Hu LL, Suo YL, Zhang L, Jin F, Feng XA, Teng N and Li Y (2014). Effects of dietary supplementation of quercetin on performance, egg quality, cecal microflora populations, and antioxidant status in laying hens. Poultry Science, 93: 347-353. DOI: https://www.doi.org/10.3382/ps.2013-03225

Malayoglu HB, Baysal Ş, Misirlioğlu Z, Polat M, Yilmaz H and Turan N (2010). Effects of oregano essential oil with or without feed enzymes on growth performance, digestive enzyme, nutrient digestibility, lipid metabolism and immune response of broilers fed on wheat-soybean meal diets. British Poultry Science, 51: 67-80.DOI: https://www.doi.org/10.1080/00071660903573702

Manafi M (2015). Comparison study of a natural non-antibiotic growth promoter and a commercial probiotic on growth performance, immune response and biochemical parameters of broiler chicks. Journal of Poultry Science, 52: 274-281. DOI: https://www.doi.org/10.2141/jpsa.0150027

Manafi M, Hedayati M and Khalaji S (2016). Effectiveness of phytogenic feed additive as alternative to bacitracin methylene disalicylate on hematological parameters, intestinal histomorphology and microbial population and production performance of Japanese quails. Asian Australasian Journal of Animal Sciences, 29: 1300-1308. DOI: https://www.dx.doi.org/10.5713%2Fajas.16.0108

McReynolds J, Waneck C, Byrd J, Genovese K, Duke S and Nisbet D (2009). Efficacy of multistrain direct-fed microbial and phytogenetic products in reducing necrotic enteritis in commercial broilers. Poultry Science, 88: 2075-2080. DOI: https://www.doi.org/10.3382/ps.2009-00106

Mitsch P, Zitterl-Eglseer K, Köhler B, Gabler C, Losa R and Zimpernik I (2004). The effect of two different blends of essential oil components on the proliferation of *Clostridium perfringens* in the intestines of broiler chickens. Poultry Science, 83: 669-675. DOI: https://www.doi.org/10.1093/ps/83.4.669

Morrissey JP and Osbourn AE (1999). Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiology and Molecular Biology Reviews**,** 63: 708-724. Available at:https://www.ncbi.nlm.nih.gov/pubmed/10477313

Murali N, Kumar-Phillips GS, Rath NC, Marcy J and Slavik MF (2012). Effect of marinating chicken meat with lemon, green tea and turmeric against food borne bacterial pathogens. International Journal of Poultry Science, 11: 326-332. DOI: http://www.dx.doi.org/10.3923/ijps.2012.326.332

Naiel MA, Ismael NE and Shehata SA (2019). Ameliorative effect of diets supplemented with rosemary (*Rosmarinus officinalis*) on aflatoxin B1 toxicity in terms of the performance, liver histopathology, immunity and antioxidant activity of Nile Tilapia (*Oreochromis niloticus*). Aquaculture, 511: 734264. DOI: https://www.doi.org/10.1016/j.aquaculture.2019.734264

Nghonjuyi NW, Tiambo CK, Kimbi HK, Manka'a CN, Juliano RS and Lisita F (2015). Efficacy of ethanolic extract of *Carica papaya* leaves as a substitute of sulphanomide for the control of coccidiosis in Kabir chickens in Cameroon. Journal of Animal Health and Production, 3: 21-27. DOI: http://www.dx.doi.org/10.14737/journal.jahp/2015/3.1.21.27

Nweze NE and Obiwulu IS (2009). Anticoccidial effects of *Ageratum Conyzoides*. Journal of Ethnopharmacology, 25: 6-9 DOI: https://www.doi.org/10.1016/j.jep.2008.11.014

Olaifa RO, Sogunle OM, Obileye LI, Ibitoye SA, Ayodeji TM, Odutayo OJ and Ogundele MA (2019). Effect of oral administration of three different phytobiotics on growth performance of locally-adapted turkeys. EC Veterinary Science, 4: 656-662. Avialable at: https://www.ecronicon.com/ecve/pdf/ECVE-04-00162.pdf

Oviedo-Rondón EO, Hume ME, Hernández C and Clemente-Hernández S (2006). Intestinal microbial ecology of broilers vaccinated and challenged with mixed *Eimeria* species, and supplemented with essential oil blends. Poultry Science, 85: 8540860. DOI: https://www.doi.org/10.1093/ps/85.5.854

Özbudak S (2019). Phytobiotics and their roles in broiler nutrition. Journal of Poultry Research, 16: 23-29. DOI: https://www.doi.org/10.34233/jpr.465575

Özek K, Wellmann KT, Ertekin B and Tarım B (2011). Effects of dietary herbal essential oil mixture and organic acid preparation on laying traits, gastrointestinal tract characteristics, blood parameters and immune response of laying hens in a hot summer season. Journal of Animal and Feed Sciences, 20: 575-586. DOI: https://www.doi.org/10.22358/jafs/66216/2011

Park IJ, Cha SY, Kang M, So YS, Go HG, Mun SP, Ryu KS and Jang HK (2011). Effect of proanthocyanidin-rich extract from *Pinus radiata* bark on immune response of specific-pathogen-free White Leghorn chickens. Poultry Science, 90: 977-982. DOI: https://www.doi.org/10.3382/ps.2010-01160

Placha I, Takacova J, Ryzner M, Cobanova K, Laukova A, Strompfova V, Venglovska K and Faix S (2014). Effect of thyme essential oil and selenium on intestine integrity and antioxidant status of broilers. British Poultry Science, 55: 105-114. DOI: https://www.doi.org/10.1080/00071668.2013.873772

Pourali M, Mirghelenj SA and Kermanshahi H (2010). Effects of garlic powder on productive performance and immune response of broiler chickens challenged with Newcastle disease virus. Global Veterineria, 4: 616-621. Available at: http://www.idosi.org/gv/gv4(6)10/16.pdf

Pourhossein Z, Qotbi AAA, Seidavi A, Laudadio V, Centoducati G and Tufarelli V (2015). Effect of different levels of dietary sweet orange (*Citrus sinensis*) peel extract on humoral immune system responses in broiler chickens. . Animal Science Journal, 86: 105-110. DOI: https://www.doi.org/10.1111/asj.12250

Radwan N, Hassan RA, Qota EM and Fayek HM (2008). Effect of natural antioxidant on oxidative stability of eggs and productive and reproductive performance of laying hens. International Journal of Poultry Science, 7: 134-150. DOI: http://www.dx.doi.org/10.3923/ijps.2008.134.150

Shad AA, Ahmad S, Ullah R, AbdEl-Salam NM, Fouad H, Ur Rehman N, Hussain H and Saeed W (2014). Phytochemical and biological activities of four wild medicinal plants. Scientific World Journal, 2014: 857363. DOI: www.dx.doi.org/10.1155/2014/857363

Si W, Ni X, Gong J, Yu H, Tsao R, Han Yand Chambers JR (2009). Antimicrobial activity of essential oils and structurally related synthetic food additives towards *Clostridium perfringens*. Journal of Applied Microbiology, 106: 213-220. DOI: https://www.doi.org/10.1111/j.1365-2672.2008.03994.x

Siragusa GR, Haas GJ, Matthews PD, Smith RJ, Buhr RJ, Dale NM and Wise MG (2008). Antimicrobial activity of lupulone against *Clostridium perfringens* in the chicken intestinal tract jejunum and caecum. Journal of Antimicrobial Chemotherapy**,** 61: 853-858. DOI: https://www.doi.org/10.1093/jac/dkn024

Sultan R, Aslam A, Saleem G, Anjum A, Krull W, Kumosani T and Barbour B (2017). Studies on performance, immunity, and safety of broilers vaccinated with killed H9N2 vaccine and supplemented with essential oils of Mentofin® in drinking water. International Journal of Applied Research and Veterinary Medicine, 15: 76-84. Available at: https://www.jarvm.com/articles/Vol15Iss2/Vol15%20iss2Barbour.pdf

Tabatabaei SN (2016). Effect of olibanum (*Boswellia thurifera*) as a feed additive on performance, some blood biochemical and intestinal morphology in broiler chicks. Research Opinions in Animal and Veterinary Sciences, 6: 130-134. Available at: http://www.roavs.com/pdf-files/Issue-4-2016/130-134.pdf

Tagoe DNA, Nyarko HD and Akpaka R (2011). A comparison of the antifungal properties of onion (*Allium cepa*), ginger (*Zingiber officinale*) and garlic (*Alliu msativum*) against *Aspergillus flavus*, *Aspergillus niger* and *Cladosporium herbarum*. Research Journal of Medicinal Plant, 5: 281-287. DOI: http://www.dx.doi.org/10.3923/rjmp.2011.281.287

Tiihonen K, Kettunen H, Bento M H L, Saarinen M, Lahtinen S, Ouwehand AC, Schulze H and Rautonen N (2010). The effect of feeding essential oils on broiler performance and gut microbiota. British Poultry Science, 51: 381-392. DOI: https://www.doi.org/10.1080/00071668.2010.496446

Tollba AAH, Shabaan SAM and Abdel-Mageed MAA (2012). Effects of using aromatic herbal extract and blended with organic acids on productive and physiological performance of poultry. Egyptian Poultry Science Journal, 30: 229-248. Available at: http://www.epsaegypt.com/pdf/2010_march/12-%201179.pdf

Tsinas A, Giannenas I, Voidarou C, Tzora A and Skoufos J (2011). Effects of an oregano based dietary supplement on performance of broiler chickens experimentally infected with *Eimeria Acervulina* and *Eimeria Maxima*. Journal of Poultry Science, 48: 194-200. DOI: https://www.doi.org/10.2141/jpsa.010123

Wang L, Piao XL, Kim SW, Piao XS, Shen YB and Lee HS (2008). Effects of *Forsythia suspensa* extract on growth performance, nutrient digestibility, and antioxidant activities in broiler chickens under high ambient temperature. Poultry Science, 87: 1287-1294. DOI: https://www.doi.org/10.3382/ps.2008-00023

Willis WL, Wall DC, Isikhuemhen OS, Jackson JN, Ibrahim SA, Hur SL and Anike F (2013). Effect of level and type of mushroom on performance, blood parameters and natural coccidiosis infection in floor-reared broilers. . Open Mycology Journal, 7: 1-6. DOI: http://www.dx.doi.org/10.2174/1874437001307010001

Windisch W, Schedle K, Plitzner C and Kroismayr A (2008). Use of phytogenic products as feed additives for swine and poultry. Journal of Animal Science, 86: 140-148. DOI: https://www.doi.org/10.2527/jas.2007-0459

Xue M, and Meng XS (1996). Review on research progress and prosperous of immune activities of bioactive polysaccharides. Journal of Traditional Veterinary Medicine, 3: 15-18. Aviable at: http://www.jstage.jst.go.jp/browse/jpsa

Yaseen SA (2003). Potential antiviral activity of *Nigella sativa* extracts.

Iraqi Journal of Veterinary Science, 17: 37-40. Available at: https://eurekamag.com/research/004/276/004276713.php

Yasodha T, Jeevitha S, Yogesh S and Ramanan M (2019). Growth performance parameters of broiler chickens as influenced by herbal poultry feed. International Research Journal of Animal and Veterinary Sciences, 1: 26-30. Available at: https://scirange.com/abstract/irjavs.2019.26.30

Youn HJ and Noh JW (2001). Screening of the anticoccidial effects of herb extracts against *Eimeria tenella*. Veterinary Parasitology, 96: 257-263. DOI: https://www.doi.org/10.1016/s0304-4017(01)00385-5

Zaki MM, Abd El-Ghany WA, Hady MM and Korany RMS (2016). Effect of certain phytobiotics on the immune response of Newcastle disease vaccinated broiler chickens Asian Journal of Poultry Science, 10: 134-140. DOI: http://www.dx.doi.org/10.3923/ajpsaj.2016.134.140

Zyan KA, Elshourbagy MA, Aggour G and Abdelfattah MA (2017). Molecular identification of *E. tenella* in broiler chicks in Kalyoubia governorate and evaluation of different strategies for control cecal coccidiosis. Benha Veterinary Medical Journal, 33: 175-182. DOI: https://www.doi.org/10.21608/BVMJ.2017.43882

Windisch W, Schedle K, Plitzner C and Kroismayr A (2008). Use of phytogenic products as feed additives for swine and poultry. Journal of Animal Science, 86: 140-148. DOI: https://www.doi.org/10.2527/jas.2007-0459

Xue M, and Meng XS (1996). Review on research progress and prosperous of immune activities of bioactive polysaccharides. Journal of Traditional Veterinary Medicine, 3: 15-18.

Yaseen SA (2003). Potential antiviral activity of *Nigella sativa* extracts. Iraqi Journal of Veterinary Science, 17: 37-40.

Yasodha T, Jeevitha S, Yogesh S and Ramanan M (2019). Growth performance parameters of broiler chickens as influenced by herbal poultry feed. International Research Journal of Animal and Veterinary Sciences, 1: 26-30. Available at: https://scirange.com/abstract/irjavs.2019.26.30

Youn HJ and Noh JW (2001). Screening of the anticoccidial effects of herb extracts against *Eimeria tenella*. Veterinary Parasitology, 96: 257-263. DOI: https://www.doi.org/10.1016/s0304-4017(01)00385-5

Zaki MM, Abd El-Ghany WA, Hady MM and Korany RMS (2016). Effect of certain phytobiotics on the immune response of Newcastle disease vaccinated broiler chickens Asian Journal of Poultry Science, 10: 134-140. DOI: http://www.dx.doi.org/10.3923/ajpsaj.2016.134.140

Zyan KA, Elshourbagy MA, Aggour G and Abdelfattah MA (2017). Molecular identification of *E. tenella* in broiler chicks in Kalyoubia governorate and evaluation of different strategies for control cecal coccidiosis. Benha Veterinary Medical Journal, 33: 175-182. DOI: https://www.doi.org/10.21608/BVMJ.2017.43882

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 580-586, December 25, 2020

Research Paper, PII: S2322455X2000066-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.66

Phenotypic Correlations among Various Egg Quality Traits in Pearl Grey, Lavender, Royal Purple, and White Varieties of Helmeted Guinea Fowl

Freddy Manyeula*, Obusitswe Tumagole, and Patrick Kgwatalala

Department of Animal Science and Production, Botswana University of Agriculture and Natural Resources, Private Bag 0027 Gaborone, Botswana

*Corresponding author`s Email: fmanyeula@buan.ac.bw; ORCID: 0000-0002-6483-4069

Received: 15 Oct. 2020
Accepted: 03 Dec. 2020

ABSTRACT

Guinea fowls are increasingly popular in Botswana since they not only provide an alternative to access protein in the form of eggs and meat but also become a good source of income for the peasants. There are different varieties of Guinea fowl in Botswana, including pearl grey, lavender, royal purple, and white. Indeed, there is a need to conduct more studies related to the phenotypic correlations among egg quality traits in different varieties of helmeted guinea fowl found in Botswana. Therefore, the present study was targeted toward the evaluation of both the external and internal quality characteristics of the four different varieties of the domesticated helmeted guinea fowl found in Botswana. In this regard, a total of 150 eggs were collected from the so-called varieties of domesticated helmeted guinea fowl. The egg weight was positively and significantly correlated with egg length, egg width, shell weight, egg surface area, and egg volume; however, the egg weight was negatively correlated with egg shape index. Of the four varieties of domesticated helmeted guinea fowl found in Botswana, the white variety had the strongest correlation coefficients with various external egg quality traits and different internal egg quality characteristics. The lavender variety had the highest correlation coefficients with internal and external egg quality traits. It seems that the selection for higher egg weight as is the case in the current egg grading system can lead to the greatest improvements in other egg quality characteristics in the white and lavender varieties, compared to the pearl grey and royal purple varieties. Therefore, the lavender and white varieties are the potential candidates for the possible selection of layer-type guinea fowl varieties.

Keywords: Botswana, Egg traits, Guinea fowl, Layer-type

INTRODUCTION

Guinea fowl production in Botswana is still at its early developmental stage and presents a viable alternative for the diversification of the country's poultry sector (Kgwatalala et al., 2013). Guinea fowl production has great potential because guinea fowl meat is an acceptable food product and its consumption is not restricted by any traditional or religious taboos (Alkan et al., 2013). Some challenges facing guinea fowl production in Botswana include poor management skills by farmers, insufficient technical and financial support from government extension services (Moreki and Seabo, 2012). Guinea fowl provide an alternative for the rural population in Botswana to access protein in the form of meat and eggs and have great potential for revenue generation through sales of live birds and eggs (Kgwatalala et al., 2013). Guinea fowl production could be a viable alternative to failed commercial chicken enterprises in rural areas of Botswana since guinea fowls are tolerant of poultry diseases compared to chickens (Moreki and Seabo, 2012). In Africa, guinea fowl production is practiced on a small scale due to the poor performance of native genotypes, and lack of information regarding genetic and phenotypic differences in traits of economic (Alkan et al., 2013). Apart from guinea fowl meat, guinea fowls also produce eggs which are often sold hard-boiled in local markets in many African countries (Adeyeye, 2012) or as fresh eggs for artificial incubation and hatching. Understanding the structure of the egg and its various components is essential for understanding egg quality, egg fertility, embryo

development, and diseases of poultry (Islam and Duta, 2010). Phenotypic correlations among various egg quality characteristics are useful in predicting the consequences of selection for a particular trait on other traits of economic importance. It seems that currently there is very limited work on phenotypic correlations among various egg quality characteristics in different varieties of helmeted guinea fowl found in Botswana. The purpose of the current study was therefore to compute correlations among various egg quality traits in pearl grey, royal purple, white, and lavender varieties of helmeted guinea fowl found in Botswana.

MATERIALS AND METHODS

Location and duration of the study

The study was conducted at the Botswana University of Agriculture and Natural Resources (BUAN), Content Farm, Sebele, Gaborone, in the Southern part of Botswana. BUAN is located at 25.94^0S, 24.58^0E at an altitude of 991 meters. The study commenced at the beginning of January 2018 and ended in the middle of March 2018.

Housing and management

The samples in this study were divided into 30 females and 5 males of pearl grey variety, 30 females and 5 males of royal purple variety, 30 females and 5 males of the white variety, and finally 30 females and 5 males of lavender variety. The four varieties of guinea fowl were raised under an intensive management system in four separate deep litter houses with concrete floors covered with wood shavings. The four deep litter houses were of similar dimensions and measured 9m x 4m. The guinea fowls were fed commercial broiler grower pellets and provided with water *ad libitum* throughout the study period. The guinea fowl were of the same age and the egg collection began when guinea fowl were 25 months of age and lasted for 7 days. The guinea fowl were raised under natural light (12hr light and 12hr dark periods) throughout the study phase.

Collection of eggs

A total of 150 eggs were collected from each variety of domesticated helmeted guinea fowl for the evaluation of both external and internal egg quality characteristics. The guinea fowl providing the eggs were of the same age (25 months) and were hatched on the same day via artificial incubation.

Measurements of egg quality traits

The investigated external egg quality characteristics included egg weight (g), egg length (cm), egg width (cm), egg surface area (cm^3), shell thickness (mm), and egg shape index (%). The examined internal egg quality characteristics entailed yolk weight (g), yolk width (cm), yolk height (cm), fresh albumin weight (g), albumin index (%), albumin (%), yolk (%), and yolk index (%). Egg weight was determined using an electronic balance, while egg length and egg width were determined using an electronic vernier caliper. Eggshell thickness was determined using a micrometer screw gauge calibrated in mm. The accuracy of shell thickness was ensured by measuring shell thickness at the broad end, a middle portion, and narrow end of the shell and taking the average of the three measurements.

Statistical analysis

The different external and internal egg characteristics of various varieties of the helmeted guinea fowl were evaluated using correlation procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA, SAS, 2010). The correlations procedure of SAS automatically tested for a significant association between various pairs of traits at $P \leq 0.05$.

Ethical approval

The rearing of the research birds was approved by the Animal Research Ethics Committee of the Botswana University of Agriculture and Natural Resources (Approval No. 2020-10), which conforms to the guidelines and the use of research animals.

RESULTS AND DISCUSSION

Phenotypic correlations among external egg quality traits

Significant positive correlations were observed between egg weight and other external egg quality traits, such as egg length (r = 0.71, 0.74, 0.64, and 0.78), egg width (r = 0.79, 0.81, 0.83, and 0.87), and egg surface area (r = 0.95, 0.89, 0.92, and 0.97) in pearl grey, lavender, royal purple and white varieties of domesticated helmeted guinea fowl, respectively (Table 1). The correlation coefficients between egg weight and shell weight were both significant ($p \leq 0.05$) and moderate (r = 0.42, 0.58, 0.35, and 0.69) in pearl grey, lavender, royal purple, and white varieties of domesticated helmeted guinea fowl, respectively. Moreover, significant positive correlations between egg weight, egg length, and egg width in different

varieties of guinea fowl were consistent with the obtained results of the studies conducted by Alkan et al. (2013) and Madibela et al. (2012). Regarding helmeted guinea fowl, egg weight, egg length, and egg width had higher correlation coefficients in this study were higher, compared to the obtained results of the same traits reported by Madibela et al. (2012). The white variety had the highest correlation coefficient of egg weight with length, width, surface area, and shell weight of the four varieties of helmeted guinea fowl. Therefore, the selection for increased egg weight (current egg grading system based on egg weight) can lead to the greatest improvements in egg length, width, surface area, and shell weight in the white variety, compared to the other three varieties.

Table 1. Phenotypic correlations among egg quality traits in pearl grey, lavender, royal purple, and white varieties of domesticated helmeted guinea fowl

Varieties	TRAIT	EW	EL	EWD	ESI	AWT	YWT	EC	SWT	STH	ESA	EV
PG	EW	1.000	0.713*	0.790*	-0.169	0.092	0.125	0.130	0.419*	-0.057	0.951*	0.967*
L		1.000	0.744*	0.805*	-0.257*	0.040	0.344*	0.203	0.584*	0.045	0.892*	0.908*
RP		1.000	0.640*	0.830*	-0.099	0.018	-0.132	-0.060	0.345*	0.020	0.919*	0.968*
W		1.00	0.776*	0.870*	-0.086	-0.156	-0.194	-0.176	0.685*	0.275	0.972*	0.979*
PG	EL		1.000	0.203*	-0.796*	0.013	0.078	0.047	0.191	-0.149	0.852*	0.685*
L			1.000	0.487*	-0.779*	-0.080	0.206	0.046	0.446*	0.020	0.913*	0.818*
RP			1.000	0.173	-0.820*	0.055	-0.136	-0.035	0.376*	-0.056	0.864*	0.699*
W			1.00	0.467*	-0.680*	-0.161*	-0.209	-0.184	0.744*	0.299	0.867*	0.740*
PG	EWD			1.000	0.430*	0.104	0.129	0.141	0.262*	-0.026	0.685*	0.852*
L				1.000	0.186	0.175	0.357*	0.306*	0.442*	0.088	0.787*	0.890*
RP				1.000	0.414*	0.063	0.067	0.083	0.124	0.004	0.645*	0.824*
W				1.00	0.383*	-0.061	-0.141	-0.086	0.439*	0.198	0.813*	0.917*
PG	ESI				1.000	0.052	-0.001	0.039	-0.018	0.122	-0.363*	-0.106
L					1.000	0.215	0.037	0.174	-0.185	0.043	-0.458*	-0.280
RP					1.000	-0.025	0.111	0.043	-0.274*	0.063	-0.425*	-0.171
W					1.00	0.114	0.102	0.118	-0.408*	-0.159	-0.225	-0.013
PG	AWT					1.000	0.256*	0.890*	0.137	0.039	0.064	0.081
L						1.000	0.306*	0.877*	0.012	0.048	0.028	0.074
RP						1.000	0.211	0.841*	0.030	0.102	0.079	0.086
W						1.00	0.704*	0.986*	-0.121	-0.339	-0.152	-0.135
PG	YWT						1.000	0.669*	0.114	-.0.169	0.127	0.135
L							1.000	0.726*	0.249	-0.055	0.311*	0.338*
RP							1.000	0.707*	0.035	0.106	-0.089	-0.049
W							1.000	0.827*	-0.100	-0.141	-0.224	-0.208
PG	EC							1.000	0.159	-0.500	0.109	0.126
L								1.000	0.134	0.006	0.177	0.224
RP								1.000	0.041	0.132	0.007	0.035
W								1.000	-0.122	-0.306	-0.180	-0.163
PG	SWT								1.000	0.384*	0.280*	0.292*
L									1.000	0.133	0.517*	0.519*
RP									1.000	0.503*	0.359*	0.313*
W									1.000	0.522*	0.715*	0.646*
PG	ESA										1.000	0.965*
L											1.000	0.981*
RP											1.000	0.964*
W											1.000	0.977*

*: $P \leq 0.05$; PG: Pearl grey; L: Lavender; RP: Royal purple; W: White varieties; EW: Egg weight; EL: Egg length; EWD: Egg width; ESI: Egg shell index; AWT: Albumin weight; YWT: Yolk weight; EC: Egg content; SWT: Shell weight; STH: Shell thickness; ESA: Egg surface area; EV: Egg volume.

Negative correlations were recorded between egg weight and egg shape index in all the four varieties of helmeted guinea fowl and between egg weight and shell thickness in the pearl grey variety. Islam and Dutta (2010) also reported negative correlations between egg weight and shape index (r= -0.17, -0.21, -0.21, -0.49, 0.05) in indigenous, exotic, and crossbred chickens of Rajshahi, Bangladesh. A negative association between egg weight and egg shape index has also been reported in other poultry species (Dzungwe et al., 2018; Godson et al., 2020). Duman et al. (2016) however reported a significant positive association between egg shape index and egg weight implying that heavier eggs are rounder in shape than lighter eggs. A negative association between egg weight and eggshell thickness suggests that heavier eggs are more thin-shelled and therefore more prone to breakages than lighter eggs. Strong, significant, and positive correlation coefficients between egg weight and other egg quality parameters indicated that selection for higher egg weight in guinea fowls might lead to simultaneous positive improvements in egg length, egg width, and egg surface area. However, it might negatively affect eggshell thickness which would lead to large eggs that are more prone to breakages.

Egg length had a significant positive association with egg surface area (r = 0.85, 0.91, 0.86, 0.87), egg volume (r = 0.69, 0.82, 0.70, 0.74), and weak positive association with egg width (r = 0.20, 0.49, 0.17, 0.47) in the pearl grey, lavender, royal purple and white varieties of domesticated helmeted guinea fowl, respectively. Weak correlation coefficients between egg length and egg width in all the four varieties of domesticated helmeted guinea fowl found in the current study were consistent with a study performed by Madibela et al. (2012) who reported a weak positive correlation between egg length and width (r = 0.25) in guinea fowls. Yakubu et al. (2008) found a strong and positive association correlation between egg length and width (r=0.71) in Nigerian indigenous chickens. Alkan et al. (2013) also contented moderate and positive correlation coefficients between egg length and width in pearl grey and royal purple varieties of helmeted guinea fowl. Egg surface area was also observed to have a high, significant ($p \leq 0.05$) and positive correlation coefficient with egg volume (r = 0.97, 0.98, 0.96, 0.98). Of the four varieties, the lavender variety had the highest correlation coefficient. Significant correlation coefficients between egg length, width, and surface were due to the fact that egg length and width can determine the volume and holding capacity of an egg, which in turn gives an indication of egg surface area and egg weight (Alkan et al., 2013). This implies that direct selection for either egg length or width will result in simultaneous improvement in egg volume, surface area, and weight. A weak, positive, and non-significant correlation coefficient was observed between egg length and eggshell weight in the pearl grey variety (r=0.19) while other varieties had moderate and significantly positive correlation coefficients. Negative and non-significant correlation coefficients were observed between egg length and eggshell thickness in pearl grey and royal purple varieties (r=-0.15 and -0.06, respectively) while other varieties were of weak, positive, and non-significant correlation coefficients. Strong, positive, and significant correlation coefficients existed among egg width, surface area, and volume in all four varieties indicating that egg surface area and volume increase with the growth of egg width. Consequently, the direct selection for egg width will result in simultaneous improvement in egg surface area and volume. There was a weak to moderate and significant correlation coefficient between egg width and shell weight in all the varieties of helmeted guinea fowl except for the royal purple which had a weak non-significant correlation coefficient. Weak to moderate correlation coefficients between egg width and shell weight observed in the four varieties of guinea fowl were consistent with Yakubu et al. (2008) who reported a weak association between egg width and shell weight (r=0.19) in Nigerian indigenous chicken. A moderate, positive and significant correlation between egg width and egg shape index occurred in the pearl grey, royal purple, and white varieties (r=0.43, 0.41, and 0.38, respectively) while a weak, positive and non-significant correlation coefficient was observed between the two traits in the lavender variety. Negative, moderate, and significant correlations occurred between egg shape index and surface area in the different varieties of domesticated helmeted guinea fowl except for the white variety which showed a negative but non-significant correlation coefficient (r= -0.23). Ultimately, weak, positive and non-significant correlation coefficients were also observed between egg shape index and shell thickness in the different varieties of domesticated helmeted guinea fowl except white variety which indicated a negative but non-significant correlation coefficient (r=-0.16).

Phenotypic correlations among internal egg quality traits

Strong, positive, and significant correlations occurred between albumin weight and egg content (r = 0.89, 0.88, 0.84, and 0.99), and weak non-significant correlations were observed between albumin weight and

egg volume (r = 0.08, 0.07, 0.07, and -0.14) in the pearl grey, lavender, royal purple and white varieties of guinea fowl, respectively. Weak, positive, and significant correlations occurred between albumin weight and yolk weight (r = 0.26 and 0.31) in pearl grey and lavender variety**,** respectively. The correlation coefficients between albumin weight and yolk weight in pearl grey and lavender were consistent with Udoh et al. (2012) who also recorded a weak, positive, and significant correlation between albumin weight and yolk weight in naked neck local chickens (r = 0.28). Yolk weight was strongly, positively, and significantly correlated with egg content (r = 0.67, 0.73, 0.71, and 0.83) in the pearl grey, lavender, royal purple, and white varieties. However, negative, non-significant correlation coefficients were observed between yolk weight and egg volume in the pearl grey and white varieties (r = -0.14 and -0.05, respectively). In addition, a weak, positive, and significant correlation coefficient was observed between the two traits in the lavender variety. The most desirable egg should preferably have more albumin weight (more protein) and less yolk weight (less fat).

Phenotypic correlations between external and internal egg quality traits

There was a strong, positive, and significant correlation between egg weight and volume (r = 0.97, 0.91, 0.97 and 0.98), while egg weight showed weak, non-significant, both negative and positive correlations with other internal quality traits, such as albumin weight (r = 0.09, 0.04, 0.02 and -0.16), yolk weight (r = 0.13, 0.34, -0.13 and -0.19), and egg content (r = 0.13, 0.20,-0.06 and -0.18) in pearl grey, lavender, royal purple, and white varieties of guinea fowl, respectively. A strong correlation coefficient between egg weight and volume was observed in all the four varieties of guinea fowl that were consistent with the reported findings of Islam and Dutta (2010) who mentioned a strong positive correlation coefficient between egg weight and volume (r = 0.93) in the Sonali chicken breed of Bangladesh. The white variety had the highest correlation coefficient between egg weight and volume, while the pearl grey had the highest correlation coefficient between egg weight and albumin weight.

Strong, positive, and significant ($p \leq 0.05$) correlations existed between egg length and volume (r = 0.69, 0.82, 0.70, and 0.74) in the pearl grey, lavender, royal purple, and white varieties of guinea fowl, respectively. Weak and positive correlations (r = 0.05 and 0.05) were observed between egg length and content in pearl grey and lavender varieties, respectively, and negative correlations (-0.04 and -0.18) were observed between egg length and egg content in royal purple and white varieties, respectively. All the correlation coefficients between egg length and egg content were non-significant in all the four varieties of helmeted guinea fowl. Weak, non-significant, positive correlations existed between egg width and albumin weight (r = 0.10, 0.18, 0.06 and -0.06), egg content (r = 0.14, 0.31, 0.08 and -0.09) and egg yolk weight (r = 0.13, 0.36, 0.07 and -0.14) while strong, positive and significant correlations were observed between egg width and egg volume (r = 0.85, 0.89, 0.82 and 0.92) in the pearl grey, lavender, royal purple and white varieties of helmeted guinea fowl, respectively. Weak, positive correlation coefficients between egg width and albumin weight observed in all the four varieties of guinea fowl were not in the same line with the obtained results of a study conducted by Yakubu et al. (2008) indicating a moderate, positive, and significant correlation coefficient between egg width and albumin weight (r = 0.57) in the free-range naked neck and full feathered chickens. Weak, non-significant positive correlation coefficients existed between egg surface area and albumin weight (r= 0.06, 0.03, 0.08, and -0.15), egg content (r=0.11, 0.18, 0.007, and -0.18) and yolk weight (r= 0.13, 0.31, -0.09, and -0.22) while strong, positive and significant correlations existed between egg surface area and volume (r = 0.97, 0.98, 0.96, and 0.98) in the pearl grey, lavender, royal purple and white varieties of helmeted of guinea fowl, respectively.

Egg volume was weakly and positively correlated with albumin weight (r = 0.08, 0.07, 0.07 and -0.14), egg content (0.13, 0.22, 0.04 and -0.16), and yolk weight (0.14, 0.34, -0.05 and -0.21) in the pearl grey, lavender, royal purple and white varieties of guinea fowl, respectively. Weak, negative, and non-significant correlations existed between egg shape index and egg volume (r = -0.11, -0.28, -0.17, and -0.01) while weak, positive, and non-significant correlations existed between egg shape index and egg content (0.04, 0.17, 0.04, and 0.12) in the pearl grey, lavender, royal purple and white varieties of helmeted guinea fowl, respectively. Egg shape index was weakly, positive to negative, and non-significantly correlated with albumin weight (r = 0.05, 0.22, -0.03 and 0.11) and yolk weight (r = - 0.001, 0.04, 0.11, and 0.10) in the pearl grey, lavender, royal purple and white varieties of domesticated helmeted guinea fowl, respectively. Weak, positive, and non-significant correlation coefficients between egg shape index and albumin weight were consistent with Islam and Dutta (2010) who also reported weak, positive, and non-

significant correlation coefficients between the two traits in five genetic groups of chicken in Rajshahi (r = 0.27, 0.10, -0.15, -0.44 and -0.13), respectively.

Weak, positive, and non-significant correlations existed between shell weight and albumin weight, yolk weight, and egg content in all the four varieties of helmeted guinea fowl. Weak and moderate, positive, and significant correlations were observed between shell weight and egg volume (r = 0.29, 0.52, 0.31, and 0.65) in the pearl grey, lavender, royal purple, and white guinea fowl varieties, respectively. Shell thickness was weakly and non-significantly correlated with egg content and yolk weight in all the varieties of helmeted guinea fowl.

The lavender variety had the highest correlation coefficient between various internal and external egg quality characteristics. It had the highest correlation coefficients with egg width and albumin weight, egg content, and yolk weight. The lavender variety also exhibited higher correlation coefficients between egg surface area and egg volume, egg content, and yolk weight as well as between egg volume and egg content and yolk weight. Improvements in external egg quality characteristics (egg weight, egg length, egg width) in guinea fowl are, therefore, likely to also lead to the greatest improvements in internal egg quality traits in lavender than other guinea fowl varieties.

CONCLUSION

Selection for higher egg weight in guinea fowl may lead to simultaneous improvements in egg length, width, shell weight, surface area, and volume. It might, however, negatively affect the egg shape index and shell thickness in all the four varieties of domesticated helmeted guinea fowl. Among the four varieties of helmeted guinea fowl, the white variety had the strongest correlation coefficients with various external egg quality traits and various internal egg quality characteristics. The lavender variety had the highest correlation coefficients with internal and external egg quality characteristics. The white and lavender guinea fowl varieties will therefore be more responsive than the pearl and royal purple varieties for selection of egg-type or layer type guinea fowl.

DECLARATIONS

Acknowledgements

The authors would like to thank the Botswana University of Agriculture and Natural Resources for funding the study.

Competing interests

The authors declare that they have no competing interest

Author's contributions

P Kgwatalala designed the research. F Manyeula and O Tumagole performed the research and wrote the manuscript. All authors read and approved the final version of the manuscript.

REFERENCES

Adeyeye EI (2012). Evaluation of the amino acid profile of the yolk and albumen of guinea fowl (Numida meleagris) egg. Applied Biology, 47: 8799-8803.

Alkan S, Karsli T, Galiç A and Karabağ K (2013). Determination of phenotypic correlations between internal and external quality traits of guinea fowl eggs. Kafkas Universitesi Veteriner Fakultesi Dergisi, 19 (5): 861-867. DOI: https://doi.org/10.9775/KVFD.2013.8988

Duman MA, Sekeroglu A, Yildirim A, Elroglu H and Camci O (2016). Relation between egg shape index and egg quality characteristics. European of Poultry Science, 80: 2-10. DOI: https://doi.org/10.1399/eps.2016.117

Dzungwe JT, Gwaza DS and Egahi JO (2018). Phenotypic correlation between egg weight and egg linear measurements of the French broiler guinea fowl raised in the humid zone of Nigeria, Current Trends on Biostatistics and Biometrics 1: 22-25. DOI: https://doi.org/10.32474/CTBB.2018.01.000104

Godson A, Gabriel A, Mark ES, Theresah N and David A (2020). Correlation between egg weight and egg characteristics in Japanese quail. International Journal of Animal Science and Technology, 8: 51-54. DOI: https://doi.org/10.11648/j.avs.20200803.11

Islam MS and Dutta RKJ (2010). Egg quality traits of indigenous, exotic and crossbred chickens (*Gallus domesticus*) in Rajshahi, Bangladesh. Journal of Life Earth Science, 5: 63-67. DOI: https://doi.org/10.3329/jles.v5i0.7352

Kgwatalala PM, Bolebano L and Nsoso SJ (2013). Egg quality characteristics of different varieties of helmeted Guinea fowl (Numida meleagris). International Journal of Poultry Science, 12 (4): 245-250. DOI: https://doi.org/10.3923/ijps.2013.245.250

Madibela OR, Moreki JC and Tebesi T (2012). Effects of storage time on internal and external characteristics of Guinea fowl (Numida meleagris) eggs. Journal of Animal Science Advances, 2 (6): 534-542. DOI: https://doi.org/10.5455/jasa.20130522044205

Moreki JC and Seabo D (2012). Guinea fowl production in Botswana. Journal of World's Poultry Research 2 (1): 1-4.

Statistical Analysis System (SAS), Base SAS 9.3. (2010). Procedures guide: 5th ed. Carry, NC: SAS Institute Inc.

Statistical procedures: SAS Institute Inc. Available at: http://support.sas.com

Udoh UH, Okon B and Udoh AP (2012). Egg quality characteristics, phenotypic correlations and prediction of egg weight in three (naked neck, frizzled feather and normal feathered) Nigerian local chickens. International Journal of Poultry Science, 11: 696-699. DOI: https://doi.org/10.3923/ijps.2012.696.699

Yakubu A, Ogah DM and Barde RE (2008). Productivity and egg quality characteristics of free range naked neck and normal feathered Nigerian indigenous chicken. International Journal of Poultry Science, 7: 579-585. DOI: https://doi.org/10.3923/ijps.2008.579.585

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 587-598, December 25, 2020

Research Paper, PII: S2322455X2000067-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.67

Polymorphism of the Prolactin Gene in Egyptian Duck Breeds

Nevien M. Sabry[1], Dalia M. Mabrouk[1], Mohamed A. Abdelhafez[1], Esteftah M. El-Komy[2] and Karima F. Mahrous[1]*

[1]*Cell Biology Department, Genetic Engineering & Biotechnology Research Division, National Research Centre, Giza 12622, Egypt*
[2]*Animal Production, Genetic Engineering & Biotechnology Research Division, National Research Centre, Giza 12622, Egypt*

*Corresponding author's Email: l_fathy@yahoo.com; ORCID: 0000-0002-1662-1034

Received: 18 Oct. 2020
Accepted: 07 Dec. 2020

ABSTRACT

In avian, the prolactin hormone triggers and regulates ovarian follicle development. This study aims to detect the Prolactin (*PRL*) gene polymorphisms (exons 1and5) in four Egyptian duck breeds, namely Campbell, Moulard, Muscovy, and Pekin using PCR-RFLP technique and sequence analysis. It also investigated the association of this gene with egg production, egg weight, and body weight. The present results revealed that *PRL* gene exon 1 and part of intron 1 showed two alleles A and B (polymorphic) in each of Campbell and Moulard, however, Muscovy and Pekin had only one allele (monomorphic). The allele A was more dominant with frequencies of 0.70, 0.60, and 1.00, compared to the allele B (0.30, 0.40, and 0.00) for Campbell, Moulard, and Muscovy, respectively. For Pekin, the allele B only appeared with the frequency of 1.0. Ducks with the high frequency of allele A were superior at egg weight, compared to others. Furthermore, for *PRL* gene exon 5, there were two alleles G and C (polymorphic) in Campbell, Moulard, and Muscovy, however, Pekin had only one allele (monomorphic). The allele G was more dominant (0.15, 0.74, 0.0, and 0.84) than the allele C (0.85, 0.26, 1.0, and 0.15) for Campbell, Moulard, Pekin, and Muscovy, respectively. Ducks having a high frequency of allele C were superior at egg production. Furthermore, there were many single nucleotide polymorphisms (SNPs) in the sequences in all breeds. The utmost ones exist at the restriction sites of *Xba*I enzyme for the amplified fragment, in the promotor, exon 1 and intron 1 (T378C in intron 1), and *Dra*I enzyme for that in exon 5 (A5871G in exon 5).

Keywords: Duck, Genetic polymorphism, Genotyping, Prolactin gene

INTRODUCTION

Meat and eggs of ducks have a high nutritional value for humans. Ducks are foremost raised in small rural farms to produce eggs and meat to improve economic livelihood. Ducks have great economic importance to many countries (Narhari, 2009). The Pekin duck (*Anas platyrhynchos domestica*), Muscovy duck (*Cairina moschata*), and Moulard ducks (crossing between Muscovy and Pekin duck), are the main duck species that are used globally to produce meat of ducks (Baeza, 2006). The Muscovy duck (*Cairina moschata*) meat has a distinct taste, with low calories, so it has global economic importance (Veeramani et al., 2016). The Muscovy and Moulard are characterized by lower fatness and higher meat production as compared by Pekin ducks (Wawro et al., 2004). The Campbell duck (*Anas platyrhynchos*) is a high egg-production breed. Moreover, the nutritional value of duck eggs is higher than chicken ones, in its mineral salts, vitamins, and amino acids (Sadar et al., 2014).

Traditional breeding approaches of selection and crossbreeding have improved the productivity of duck and devised new hybrids, while improvement using this method was slow (Asiamah et al., 2019). Duck productivity was increased using modern methods of molecular genetics, by identifying candidate genes associated with quantitative traits to improve productive traits and enhance breeding programs (Basumatary et al., 2019). One of the utmost serious problems in farming ducks is the low productivity and quality of production. Therefore, their genetic improvement aims to increase productivity, improve product quality and elevate economic value (Chang et al., 2012). Genetic improvement programs using traditional and modern methods for meat-type ducks have successfully enhanced their productive performance. In Pekin ducks, the genetically improved type for higher meat production reached to 3.2 kg at seven weeks, while unimproved duck production reached to 1.7 kg at 11 weeks of age (Zeng et al., 2016).

To cite this paper: Sabry NM, Mabrouk DM, Abdelhafez MA, El-Komy EM and Mahrous KF (2020). Polymorphism of the Prolactin Gene in Egyptian Duck Breeds. *J. World Poult. Res.*, 10 (4): 587-598. DOI: https://dx.doi.org/10.36380/jwpr.2020.67

Prolactin hormone gene (*PRL*) is a single-chain polypeptide belongs to the family of growth hormone genes, and synthesized by the anterior pituitary gland of poultry (Wang et al., 2011). The size of duck prolactin gene is 1000 bp in size and consists of five exons and four introns encoding 229 amino acids. As the chicken *PRL* gene was cloned and sequenced (Watahiki et al., 1989), a bulk of studies have targeting this gene polymorphism (Xu et al., 2015; Shamsalddini et al., 2016; Li et al., 2017). In recent years, polymorphisms in the *PRL* gene have been associated with egg production in hens (Sarvestani et al., 2013; Kulibaba, 2015; Tempfli et al., 2015; Mohamed et al., 2017; Yadav et al., 2018; Bai et al., 2019; Basumatary et al., 2019).

Identifying single nucleotide polymorphisms (SNPs) in candidate genes and their association with egg laying traits is an important technique used to enhance the avian genetics (Feng et al., 2018). Therefore, the present study used four Egyptian duck breeds; Moulard, Campbell, Pekin and Muscovy ducks to screen polymorphism of the duck *PRL* gene. Identifying of such SNPs can be employed as genetic markers for selection of genetically superior ducks to increase productivity.

MATERIALSANDMETHODS

Ethical approval

Permission for collecting the samples used in this work was received from the management of El-Nubaria farm, owned by National Research Centre that was included in this study. The samples were collected per standard sample collection procedures without any harm to animals.

Animals

The present study used a total of 60 healthy ducks, of four breeds; Campbell, Moulard, Muscovy and Pekin, raised in El-Nubaria experimental farm, owned by the National Research Centre-Giza-Egypt. 15 blood samples (3mL) were aseptically collected from each breed, using EDTA (0.5 M) as an anticoagulant.

DNA Extraction

Genomic DNA was isolated from whole blood of ducks using salting out method (Shokrzadeh and Mohammadpour, 2018), with minor modifications. Briefly, blood (3 ml) was mixed with cell lysis buffer (640 mM sucrose, 10 mM $MgCl_2$, 20 mM Tris-HCl, 2% Triton 100X) and placed on ice for 30 min then centrifuged at 5000 rpm for 15 min at 4°C and the supernatant was discarded. Nuclei lysis buffer (10 mM Tris-base, 400 mM NaCl, 2 mM EDTA) was added to the pellet and mixed with 20% Sodium Dodecyl Sulfate (SDS) and proteinase K then placed in a water bath at 55°C for two hours. NaCl (saturated) was added and centrifuged to separate a clear upper layer containing DNA. Only 30 µl of the clear layer was transferred to a tube containing absolute ethanol to fish the DNA and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA) at 37°C for 2 hours. Purity and concentration of DNA were measured using Nano Drop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Extracted DNA was stored in -20°C till usage.

Polymerase Chain Reaction (PCR)

The primers used were F: 5'-AAATTCCCTCTCACAG TTACA-3'; R: 5'-GATGCAGAGACAAGTTTCACC-3' for PRL (intron 1) to produce a product of 417 bp and F: 5'-TGCAAACCATAAAAGAAAAGA-3'; R: 5'-CAATGAAAAGTGGCAAAGCAA-3' for *PRL* (exon 5) to produce a product of 400 bp. PCR amplification was carried out in a 25 µl reaction volume, including 2 µl genomic DNA (50 ng), 2.5 µL 10X buffer, 2.5 mM of deoxyribonucleotide triphosphates (dNTPs), 2.5 µl of each primer (10 pmol), and 0.5 µl *Taq* polymerase (Thermo-Fisher Scientific, USA). The PCR conditions were; 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 30 sec at the annealing temperature 54°C, 2 min at 72°C and a final extension of 7 min at 72°C.

Restriction Fragment Length Polymorphism (RFLP)

Digestion of the PCR products was performed overnight at 37°C in a final volume of 30 µl with specific restriction enzymes (Thermo Fisher Scientific, USA); *Xba* I (5'T↓CTAGA-3') for PRL (Intron 1), and *DraI* (5 'TTTAAA↓3') for PRL (Exon 5). The RFLP reaction mixture composed of 10 µL of PCR products, 1U of restriction enzymes, 2 µl of buffer and 18 sterile H_2O. The digested products were run in 2.5% agarose gel and different genotypes were visualized under U.V. transilluminator and photographed using Gel documentation system.

DNA Sequencing and Analysis

The PCR products were purified using Gene JET Gel Extraction Kit (Thermo-Fisher Scientific, USA) according to the manufacture instructions. The purified PCR products were sequenced using an automated sequencing service (Macrogen, South Korea). Sequence analysis was

performed via program BLAST/NCBI (National Center for Biotechnology Information. http://www.ncbi.nlm.nih.gov/). Sequence alignment were accomplished by Clustal Omega version (1.2.4) (https://www.ebi.ac.uk/Tools/msa/clustalo), as described by (Larkin et al., 2007). The phylogenetic trees between the studied breeds, and other avian were done, using unweighted pair group method with arithmetic mean (UPGMA). To detect chromosomal localization of prolactin gene (exons 1 and 5) from Egyptian duck breeds in the duck genome, *Anas platyrhynchos* isolate PK-2015, IASCAAS_PekingDuck_PBH1.5, was used from NCBI.

RESULTS

In the current study, the DNA fragment (417 bp), covering the *PRL* gene promoter (220 bp), exon 1 (28 bp) and part of intron 1 (169 bp), was successfully PCR amplified, as well as the other fragment (400 bp), covers the coding region of *PRL* gene exon 5 (192 bp), and part of intron 4 (137 bp, Figure 1a).

The *Xba*I/RFLP for *PRL* (the promoter, exon 1 and part of intron 1) revealed three fragments; non-cut fragment (417 bp) or homozygous genotype (AA), cut fragments (356 bp and 61 bp) or homozygotes (BB) [61 bp band is not visible because it is too short], and the combined fragments (417 bp, 356 bp and 61 bp) or heterozygotes (AB Figure 1b). As illustrated in Table 1, both Campbell and Moulard breeds are polymorphic resulted two alleles (A and B). However, Muscovy (A) and Pekin (B) were monomorphic resulted one allele. The allele A frequency was much higher than allele B in the four breeds. The results revealed that the four investigated duck breeds, PRL gene exon 1 and part of intron 1, had two alleles (A and B) and three genotypes AA, AB, BB, ducks with higher frequency of allele A superior at egg weight, at 10-weeks-old body weight compared to other duck breeds (Table 1).

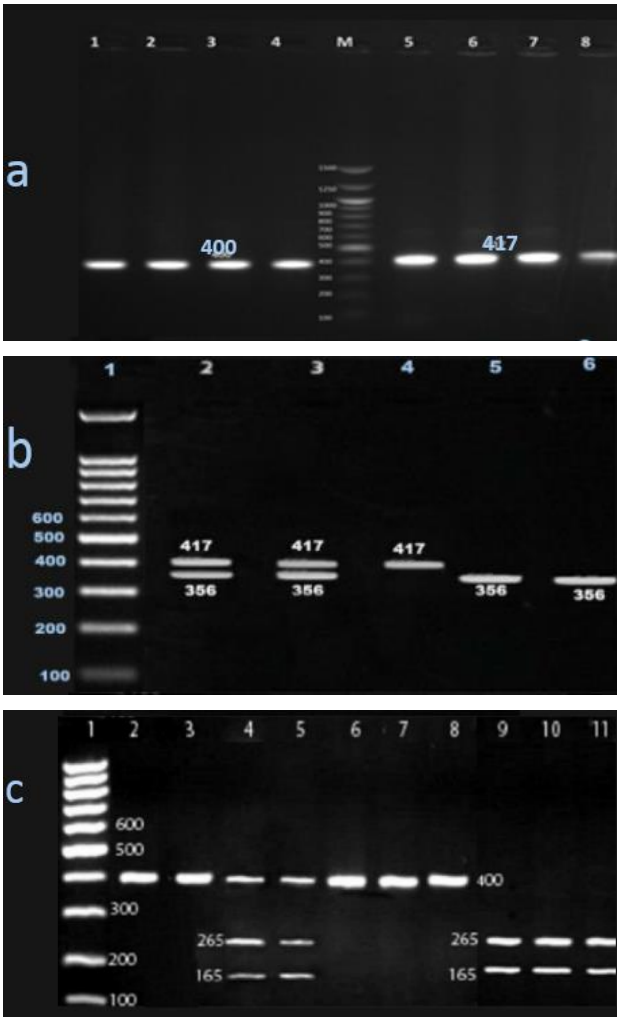


Figure 1a. Electrophoresis of the PCR products of prolactin exons 1 and 5 on agarose gel. Gel was stained with ethidium bromide, Lane 1-4: prolactin (exon5), Lanes 5-8: prolactin (exon1), Lane M: 100-bp DNA ladder. **Figure 1b.** Electrophoresis of the digestion products of *Xba*I on agarose gel. Gel was stained with ethidium bromide, Lanes 2 and 3: AB genotype, Lane 4: AA genotype, Lanes 5 and 6: BB genotype, Lane 1: 100-bp ladder. **Figure 1c.** Electrophoresis of the digestion products of *DraI* on agarose gel. Gel was stained with ethidium bromide, Lane 2, 6, 7, and 8: AA genotype, Lanes 4 and 5: AB genotype, Lanes 9, 10 and 11: BB genotype, Lane 1: 100-bp DNA Ladder.

Table 1. Relation between the four Egyptian duck breeds (Campbell, Moulard, Pekin and Muscovy) and body weight, egg number per year and egg weight, allelic and genotypic frequencies

Breed	BW10	Eggs number /year	Egg weight	Allelic Frequency				Genotypic Frequency					
				Exon 1		Exon 5		Exon 1			Exon 5		
				A	B	G	C	AA	AB	BB	GG	CG	CC
Campbell	1306.57±37.32	300-320	65 g	00.70	00.30	0.15	0.85	0.50	0.40	0.10	0.15	0.00	0.85
Moulard	4021.00±20.49	200-230	60 g	00.60	00.40	00.74	00.26	0.35	0.50	0.15	0.61	0.26	0.13
Pekin	2846.00±30.02	200-300	60 g	00.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Muscovy	3740.00±47.37	100-180	77 g	1.00	00.00	00.85	00.15	1.00	0.00	0.00	0.75	0.20	0.05

In similar manner, *Dra*I/RFLP for *PRL* (exon 5) yielded two homozygous genotypes were GG (400 bp), CC (235 and 165 bp) and heterozygotes are known as genotypes GC (400, 235 and 165 bp, Figure 1c). As illustrated in Table 1, all races are polymorphic, except Pekin which was monomorphic (C). Besides, the allele G was more dominant than allele C in the four races. Additionally, *PRL* gene exon 5 of the four manipulated duck breeds, at 10-weeks-body-old weight, had two alleles (G and C) and three genotypes GG, GC, CC, ducks that had high frequency of allele C were superior at egg production compared with the other ducks (Table 1).

Genetic polymorphisms of *PRL* gene covers the coding region of *PRL* gene (promoter, exon 1 and part of intron 1 and exon 5) between the four Egyptian duck breeds also can be detected by DNA-sequencing methods, Clustal Omega was used to aligned each breed of the four Egyptian duck breeds with each other and with *Anas platyrhynchos*, *PRL* gene, cds, AB158611 (*A. platyrhynchos*, *PRL*) presented in the database. The Moulard breed samples revealed that Moulard-1 sequences shared similarity (97.36%) with Moulard-2. Also, the DNA sequence from Campbell breed samples revealed that the Campbell-1 sequence shared similarity (97.36%) with Campbell-2. However, Moulard-1 sequences shared high similarity (100%) with Campbell-2, and Moulard-2 sequences shared high similarity (100%) with Campbell-1. The alignment between Moulard-1 and Campbell-2 with both Moulard-2 and Campbell-1 showed that Four nucleotide substitutions at nucleotide 132 (A/G), 166 (T/C), 213 (C/T) and 378 (T/C), this mutation are considered from the transition type, the other seven nucleotide substitutions at nucleotide 319, 325, 332 (T/G), 318, 322, 326, 330 (G/T) the SNPs between the sequences are considered from the transversion type.

Furthermore, *A. platyrhynchos*, *PRL* shared similarity (99.04%) with Moulard-1 and Campbell-2 sample, two nucleotide substitutions at nucleotide 267 (G/A) and at nucleotide 412 (A/G), these mutations are considered from the transition type, the other two nucleotide substitutions at nucleotides 312 (T/G) and 314 (G/T), are considered from the transversion type. Both Moulard-2 and Campbell-1 samples shared similarity (96.40%) with *A. platyrhynchos*, *PRL*, six nucleotide substitutions at nucleotide 132 (A/G), 166 (T/C), 213 (C/T), 267 (G/A), 378 (T/C) and 412 (A/G), the mutations are considered from the transition type, the other nine nucleotide substitutions at nucleotides 312, 319, 325, 332 (T/G), 314, 318, 322, 326, 330 (G/T), are considered from the transversion type (Figure 2) and percent identity matrix of the multiple sequence alignment (Table 2).

The genomic sequences of *PRL* gene from Pekin and Muscovy breeds revealed that the sequences (Pekin-1 and Pekin-2) also, (Muscovy-1 and Muscovy-2) shared high similarity (100%) between each other as there is no SNPs between the sequences. However, all Pekin breed samples shared similarity (98.32%) with *A. platyrhynchos*, *PRL* (Table 2). Eight transitions at nucleotides; 267 (G/A) and 412 (A/G). In addition, five transversions at nucleotides; 135 (T/A), 137(C/G), 213(C/T), 312 (T/G) and 314 (G/T). All Muscovy ducks shared similarity (96.40%) with *A. platyrhynchos*. Six transitions at nucleotides; 132 (A/G), 166 (T/C), 213 (C/T), 267 (G/A), 378 (T/C), 412 (A/G), and nine transversions at 312, 319, 325, 332 (T/G), 314, 318, 322, 326, 330 (G/T), were found (Figure 2).

Sequence comparisons among the four studied breeds *PRL* gene exon1 sequence revealed that Moulard-1 revealed high similarity (100%) with Campbell-2, (99.28%) with Pekin and (97.36%) with Moulard-2, Campbell-1 and Muscovy. As Muscovy resulted high similarity (100%) with Campbell-1 and Moulard-2, (97.12%) with Pekin (Table 2).

The four examined duck lines, *PRL* gene exon 5 sequence, aligned with *A. platyrhynchos*, *PRL* disclosed upraised similarity (100%) with all Pekin (Pekin-1 and Pekin-2), Moulard-2, Campbell-2 and also Muscovy-1. However, *A. platyrhynchos*, *PRL* illustrated similarity (97.25%) with Moulard-1, Campbell-1 and Muscovy-2 (Table 3). There were five transitions at nucleotides; 5780 (G/A), 5808(T/C), 5822(C/T), 5871(A/G), 5926(G/A), and six transversions at nucleotides; 5766 (G/T), 5792 (A/T), 5834 (G/T), 6004 (A/C), 6007(G/C) and 6029(G/T) (Figure 3). For the sequence comparisons among the four checked duck breeds exon 5 revealed that Moulard-1 exhibited high similarity (100%) with Campbell-1, Muscovy-2, but 97.25% with Moulard-2, Campbell-2, Muscovy-1, Pekin-1 and Pekin-2 (Table 3).

The obtained sequences were submitted to Gen bank and have been assigned with accession number; Campbell *PRL* exon1 allele A (LC565022) and allele B (LC565023), Moulard *PRL* exon 1 allele A (LC576823) and allele B (LC565024), Pekin *PRL* exon 1 (LC565026), Muscovy *PRL* exon 1 (LC565025). Also, the obtained sequences for exon 5 were submitted, Campell *PRL* exon 5 allele A (LC565015) and allele B (LC565016), Moulard *PRL* exon 5 allele A (LC565017) and allele B (LC565018), Pekin *PRL* exon 5 (LC565021), Muscovy PRL exon 5 allele A (LC565019) and allele B (LC565020).

Table 2. Percent Identity Matrix of DNA multiple sequence alignment for the four Egyptian duck breeds (Campbell, Moulard, Pekin and Muscovy) with each other and other avian species sequences of *PRL* gene exon 1 published in GenBank database

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1: *M. gallopavo*	100.00	77.27	78.64	76.50	76.50	76.50	76.92	76.50	76.50	76.50	85.88	84.71	97.37	94.12
2: *C. moschata*		100.00	91.86	98.72	98.72	98.72	97.28	97.45	97.45	97.02	90.10	86.96	87.55	86.25
3: *A. anser*			100.00	94.19	94.19	94.19	93.84	95.64	95.64	95.40	90.50	87.90	85.89	82.89
4: Campbell-1,allele A				100.00	100.00	100.00	96.40	97.36	97.36	97.12	89.30	86.75	88.44	84.73
5: Moulard-2 allele A					100.00	100.00	96.40	97.36	97.36	97.12	89.30	86.75	88.44	84.73
6: Muscovy-1,2						100.00	96.40	97.36	97.36	97.12	89.30	86.75	88.44	84.73
7: *A. platyrhynchos*							100.00	99.04	99.04	98.32	88.48	85.94	87.85	84.18
8: Campbell-2 allele B								100.00	100.00	99.28	88.89	86.35	88.00	84.36
9: Moulard-1 allele B									100.00	99.28	88.89	86.35	88.00	84.36
10: Pekin-1,2										100.00	88.48	85.94	87.56	84.00
11: *C. japonica*											100.00	97.11	96.41	91.67
12: *P. cristatus*												100.00	95.57	92.34
13: *G. gallus* B allele													100.00	94.20
14: *P. colchicus*														100.00

1: *Meleagris gallopavo PRL* AB605394, 2: *Cairina moschata PRL*, KM390982, 3: *Anser anser PRL*, GU984377, 4: Campbell-1 *PRL* Ex-1, LC565022, 5: Moulard-2 allele A, LC576823, 6: Muscovy *PRL* Ex1, LC56502, 7: *A. platyrhynchos PRL*, AB158611, 8: Campbell-2 allele B, LC565023, 9: Moulard-1 allele B, LC565024, 10: Pekin, PRL Ex-1, LC565026, 11: *Coturnix japonica PRL*, AB452962, 12: *Pavo cristatus PRL*, AB452960, 13: *Gallus gallus PRL* B allele, JN661569, 14: *Phasianus colchicus PRL*, AB452961

Table 3. Percent Identity Matrix of DNA multiple sequence alignment for the four Egyptian duck breeds (Campbell, Moulard, Pekin and Muscovy) with each other and other avian species sequences of *PRL* gene exon 5 published in GenBank database

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1: *Struthio camelus*	100.0	87.17	87.17	87.17	87.96	87.96	87.96	87.96	87.96	88.74	81.98	81.34	81.68	83.76
2: Campbell-1 alleleA_Ex5		100.00	100.00	100.00	97.75	97.75	97.75	97.75	97.75	97.50	83.89	84.20	84.48	83.54
3: Moulard-1 allele A Ex5			100.00	100.00	97.75	97.75	97.75	97.75	97.75	97.50	83.89	84.20	84.48	83.54
4: Muscovy-2 allele B Ex5				100.00	97.75	97.75	97.75	97.75	97.75	97.50	83.89	84.20	84.48	83.54
5: *A. platyrhynchos*					100.00	100.00	100.00	100.00	100.00	98.75	85.68	86.65	85.75	85.09
6: Campbell-2 allele B Ex5						100.00	100.00	100.00	100.00	98.75	85.68	86.65	85.75	85.09
7: Moulard-2 allele B Ex5							100.00	100.00	100.00	98.75	85.68	86.65	85.75	85.09
8: Muscovy-1 allele A Ex5								100.00	100.00	98.75	85.68	86.65	85.75	85.09
9: Pekin-1,2									100.00	98.75	85.68	86.65	85.75	85.09
10: *A. anser*										100.00	85.93	86.38	86.51	86.34
11*: G. gallus*											100.00	94.57	93.61	93.50
12: *P. cristatus*												100.00	94.57	94.43
13*: M. gallopavo*													100.00	98.45
14: *Ph. Colchicus*														100.00

1: *Struthio camelus PRL* AB36288, 2: Campbell-1 *PRL* gene Ex5, LC56501, 3: Moulard-1 *PRL* gene Ex5, LC565017, 4: Muscovy-2 *PRL* gene Ex5, LC565020, 5: *A. platyrhynchos PRL*, AB158611, 6: Campbell-2 *PRL* gene Ex 5, LC565016, 7: Moulard-2 *PRL* gene Ex5, LC565018, 8: Muscovy-1 *PRL* gene Ex5, LC565019, 9: Pekin *PRL* gene Ex5, LC565021, 10: *Anser anser PRL*, GU984377, 11: *Gallus gallus PRL*, AB011438, 12: *Pavo cristatus PRL*, AB605393, 13: *Meleagris gallopavo PRL*, AH005340, 14: *Phasianus colchicus PRL*, AB605395

To cite this paper: Sabry NM, Mabrouk DM, Abdelhafez MA, El-Komy EM and Mahrous KF (2020). Polymorphism of the Prolactin Gene in Egyptian Duck Breeds. *J. World Poult. Res.,* 10 (4): 587-598. DOI: https://dx.doi.org/10.36380/jwpr.2020.67

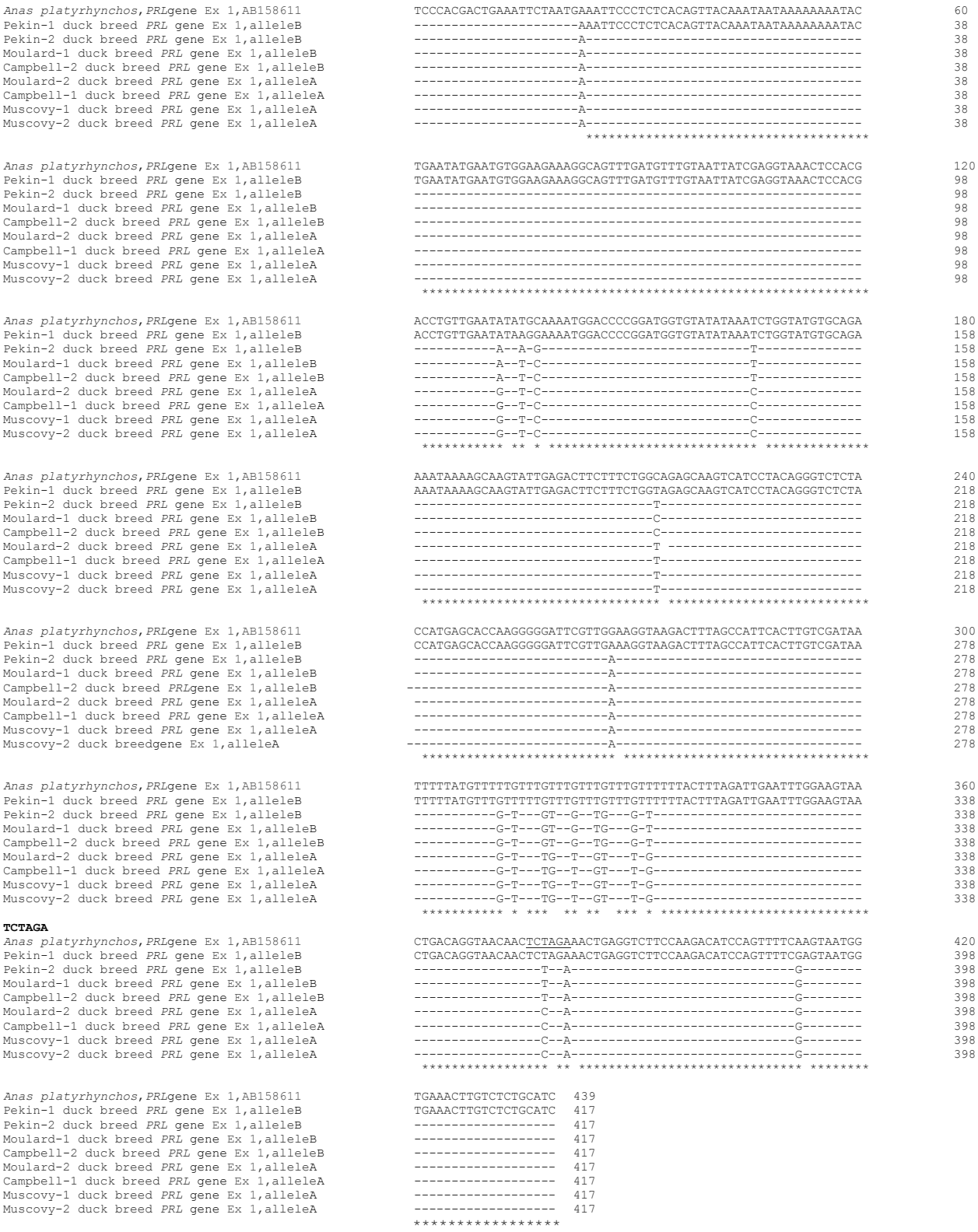


Figure 2. Multiple DNA sequence alignment of *PRL* Ex1 of the four Egyptian duck breeds (Campbell, Moulard, Pekin and Muscovy) with the *Anas platyrhynchos PRL* gene Ex 1, AB158611, and nucleotide sequences of alleles A and B showing SNPs at nt 356 and the presence of *Xba*I restriction site (T↓CTAGA) in alleles B.

To cite this paper: Sabry NM, Mabrouk DM, Abdelhafez MA, El-Komy EM and Mahrous KF (2020). Polymorphism of the Prolactin Gene in Egyptian Duck Breeds. *J. World Poult. Res.*, 10 (4): 587-598. DOI: https://dx.doi.org/10.36380/jwpr.2020.67

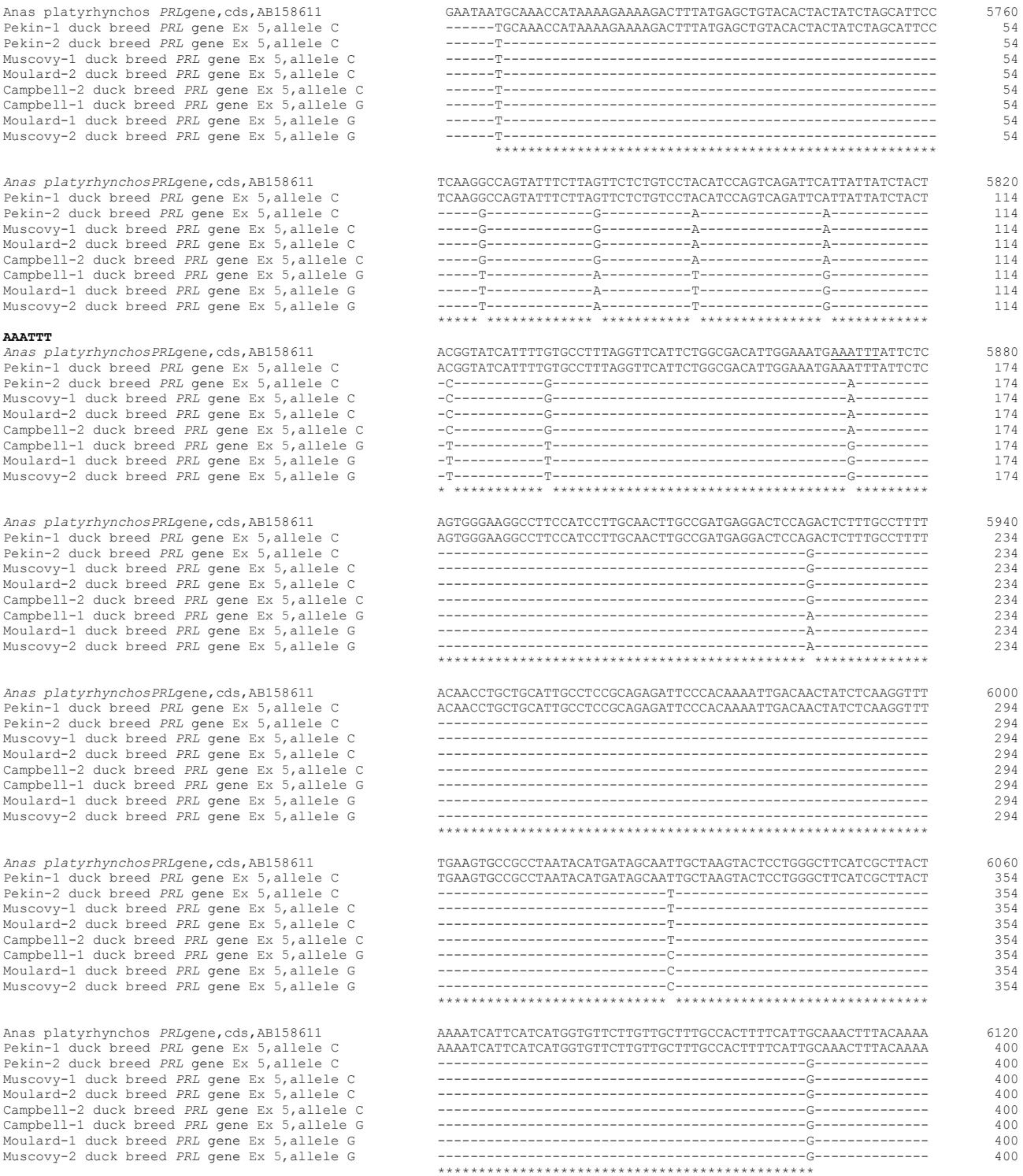


Figure 3. Multiple DNA sequence alignment of *PRL* Ex5 of the four Egyptian duck breeds (Campbell, Moulard, Pekin and Muscovy) with the *Anas platyrhynchos PRL* gene Ex 5, AB158611, and nucleotide sequences of alleles G and C showing SNPs position at nt 165 and the presence of *Dra*I restriction site (3'AAA↓TTT 5') (underlined) in alleles C.

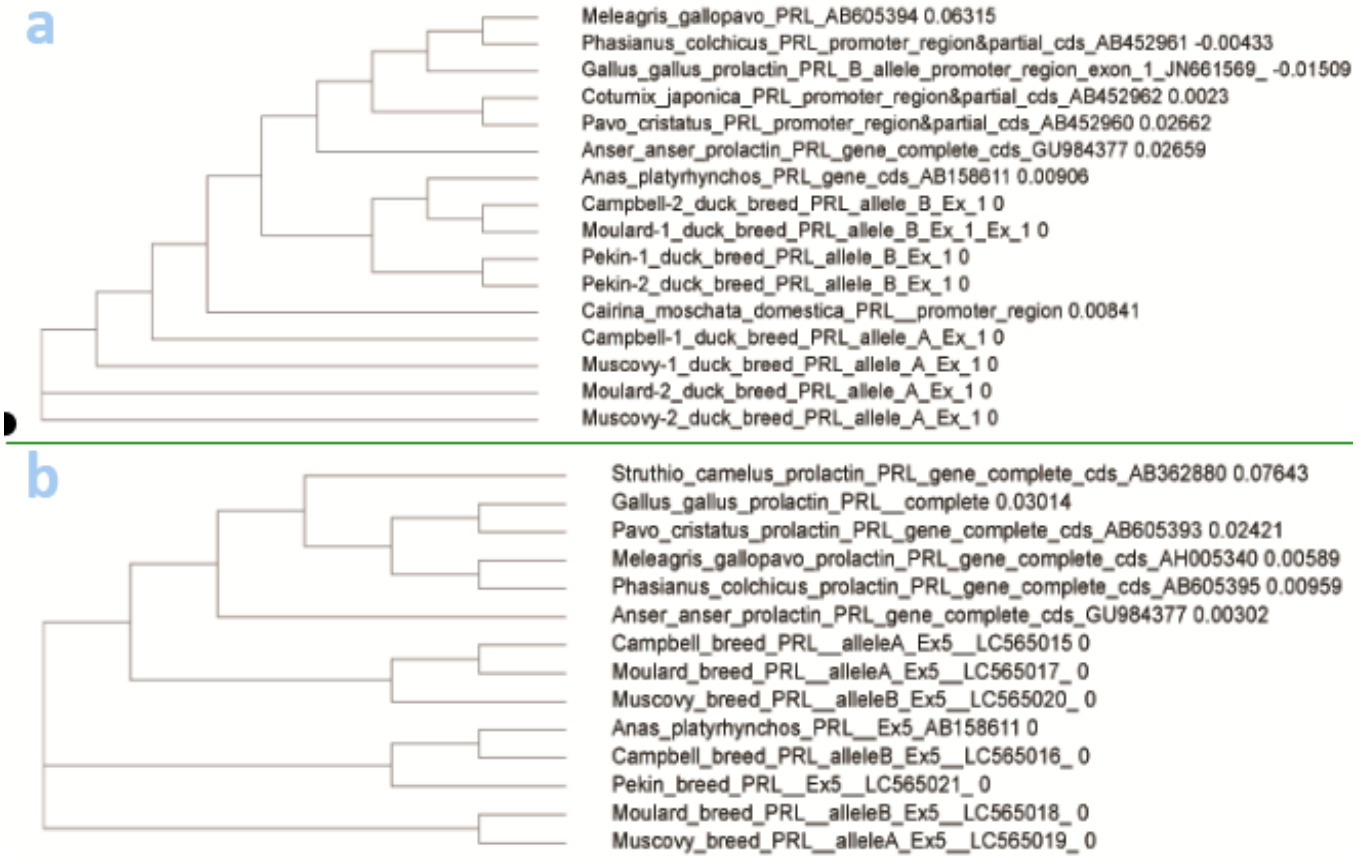


Figure 4a. Phylogenetic tree of the Multiple DNA sequence alignment of the four Egyptian duck breeds (Moulard, Pekin, Campbell and Muscovy) *PRL* gene exon 1 with the other sequences of avian prolactin gene species published in GenBank database. Figure 4b. Phylogenetic tree of the Multiple DNA sequence alignment of the Egyptian duck breed *PRL* gene exon 5 with the other sequences of avian prolactin gene species published in GenBank database.

Sequence comparison of *PRL* gene exon 1, between the four Egyptian duck breeds and other avian Species, appeared in percent identity matrix of DNA multiple sequence alignment of exon 1 (Table 2) and phylogenetic tree (Figure 4a). Likewise, DNA sequence comparison of exon 5, between the four breeds, and other birds, were illustrated in percent identity matrix of DNA multiple sequence alignment of exon 1 (Table 3) and phylogenetic tree (Figure 4b).

Regards to the phylogenetic tree, of Egyptian duck breeds, *PRL* gene exon1, the locus of Moulard-1 was related to Campbell-2 and Pekin, more than Moulard-2, Campbell-1 and Muscovy. However, the locus of Muscovy was related to Moulard-2, Campbell-1, more than Pekin (Figure 4a). For exon 5 tree, the locus of Moulard-1 was related to Campbell-2, Muscovy-2, more than Moulard-2, Campbell-2, Muscovy-1and Pekin (Figure 4b).

Lastly, prolactin gene sequence, in the four studied strains, was aligned with Duck genome, by using the BLAST program. The results of these alignments represent Duck prolactin (*PRL*) gene sequence on chromosome 2 (APL 2), *Anas platyrhynchos* isolate PK-2015 chromosome 2, IASCAAS Peking Duck PBH1.5 ranging from 49241262 to 49241678 for prolactin promoter exon 1 gene, and ranging from 49246949 to 49247348 for exon 5.

DISCUSSION

Research on the PRL gene have been widely carried out, the avian PRL gene is highly conserved and most

sequence polymorphisms in the PRL gene occur in 5′ flanking region, 3′ flanking region (Kansaku et al., 2008). The literatures focused on polymorphisms of PRL gene in 5′ flanking region (promoter region) which has been considered as an excellent experimental model for studying both tissue-specific and hormonally regulated activation of gene transcription The literature lightened up PRL gene polymorphism, in 5′ flanking region (promoter region) which has been respected as a perfect experiential example, for exploring both tissue-special and hormonally managed activation of gene transcription (Elsholtz et al., 1991; Li et al., 2009; Wang et al., 2011; Chang et al., 2012).

In this study, the results showed that Genetic polymorphisms of *PRL* gene by RFLP analysis, so the restriction enzymes *XbaI* and *DraI* have been used. The results of RFLP characterization analysis using the *Xba*I enzyme showed that the four Egyptian duck breeds PRL gene exon 1 and part of intron 1 at 10-weeks-old body weight had two alleles (A and B) and three genotypes AA, AB, BB, ducks with high frequency of allele A superior at egg weight compared to others. Into the bargain, the association of intron 1 polymorphism of *PRL* gene with egg weight was studied (Li et al., 2009) and agreed with present study. PCR-RFLP produced three genotypes AA, BB, and AB, and ducks of BB genotype were higher egg production and superior egg weight at 30 weeks than AB genotypes, which in agreement with this study. Wang et al. (2011) found variations in exons 2, 4 and 5 in local Chinese ducks, but the relationship with annual egg production was shown by exon 5. The prolactin gene SNP at intron 1, C→A mutation at position 386 at introns which can be detected by *Xba*I enzyme (Mazurowski et al., 2016), identifying two alleles G and T, and three genotypes GG, TG and TT. *PRL*/*XbaI* locus was found polymorphic in Pekin and Moulard duck populations, while monomorphic in Muscovy duck breed. Thus, the result would be useful as a control for genetic equilibrium in Muscovy ducks. In addition, Bai et al. (2019) studied the association between *PRL* gene with egg production. They found three genotypes AA, AG, GG and ducks that have GG genotype were greater in egg weight and egg production when compared to the other genotypes. Prolactin hormone gene can use as a genetic marker for reproductive traits (Bai et al., 2019; Basumatary et al., 2019). The C359A polymorphism was reported in Khaki Campbell duck as being associated with egg production at 300 days, with ducks with the GT genotype producing a greater number of eggs than those with TT and GG genotypes (Chuekwon and Boonlum, 2017).

RFLP characterization analysis results by using the *Dra*I enzyme on the exon 5 *PRL* gene fragment resulted that four Egyptian duck breed *PRL* gene exon 5 at 10-weeks-body-old weight had two alleles (G and C) and three genotypes GG, GC, CC, ducks that had high frequency of allele C were superior at egg production compared with others. Also, the results of *PRL*/*DraI* in Bayang ducks resulted three genotypes consisting of homozygotes (CC), heterozygotes (CT) and homozygotes (TT) with frequencies of 0.684, 0.293 and 0.21, respectively. The proportion of alleles obtained in the Prolactin (*PRL*|*Xba*I) gene fragments in the Bayang duck studied has a frequency of genotype and allele not much different from the genotype and allele frequency values in the study of Shaoxing local Chinese ducks (Yurnalis et al., 2019). These are in accordance with present results. The different distribution of genotypes in different duck populations may be described to the different genetic background of these populations (Wang et al., 2011). Since the chicken *PRL* gene had been cloned and sequenced (Watahikiet al., 1989), most research focused on polymorphism detection in this gene.

For genetic polymorphism of PRL gene, comprising the promoter, exon 1, part of intron 1 and exon 5, the genomic sequence comparisons between four Egyptian duck breeds *PRL* gene exon 1 sequence revealed that Moulard-1 breed sample shared high similarity (100%) with Campbell-2, (99.28%) with Pekin and (97.36%) with Moulard-2, Campbell-1 and Muscovy breed samples. As Muscovy resulted high similarity (100%) with Campbell-1 and Moulard-2, and (97.12%) with Pekin. For the *PRL* gene exon 5 the sequence comparisons revealed that Moulard-1 shared high similarity (100%) with Campbell-1, Muscovy-2, but shared similarity (97.25%) with Moulard-2, Campbell-2, Muscovy-1 and two samples of Pekin-1 and Pekin-2. Several SNPs were revealed from the both type (transition and transversion) for the two exons 1 and 5. However, the difference in genotypes between four Egyptian duck breed resulted from the presence of SNPs at T/C mutation position 378 bp in intron 1 region and A/G mutation at position 5871 bp in exon 5 region (Accession no. AB158611).

A great number of SNPs were reported in *PRL* gene for chicken. From direct sequencing and association analysis, Cui et al. (2006) got six SNPs (C-2402T, C-2161G, T-2101G, C-2062G, T-2054A and G-2040A) and a 24-bp indel (insertion-deletion) showed that the 24-bp indel was correlated to egg production and chicken

broodiness. Also, three mutations screened by Liu *et al.* (2007) (C-1607T, C-5749T and T-5821C) showed a link between different haplotypes and production of eggs. In geese, three SNPs (A-401G, G-268A and T-266A) were screened in the 5′-proximal region of *PRL* gene and statistical analysis indicated that these polymorphisms have the potential to be used for egg production in molecular breeding (Jiang et al., 2009). All the above studies showed that *PRL* is an effective candidate gene for production eggs.

Assorted gene SNPs, concerning egg-laying traits in both chicken and geese, have been exposed, with few reviews in ducks (Kang et al., 2012; Kulibaba, 2015; Alsiddig et al., 2017; Mohamed et al., 2017; Feng et al., 2018). There was a number of new SNPs, not the 12 SNPs hit in the six native Chinese duck strains (Wang et al., 2011). These results showed that the duck *PRL* gene in these duck populations is rich in polymorphisms in these duck populations.

Sequence comparisons between genomic sequence of *PRL* gene exon 1 from the four Egyptian duck lines and from the other avian species showed that sequence alignments of Moulard allele B and Campbell allele B shared similarity (99.04%, 97.45%, 95.64%, 88.00%, 76.50%, 88.89%, 86.35% and 84.36%), Muscovy exon 1, Moulard allele A and Campbell allele A shared similarity (96.40%, 98.72%, 94.19%, 88.44%, 76.50%, 89.30%, 86.75% and 84.73%) and also, Pekin exon 1 shared similarity (98.08%, 97.02%, 95.16%, 87.56%, 76.00%, 88.48%, 85.94% and 84.00%) with *PRL* gene of *A. platyrhynchos* (Moulard duck), Muscovy duck, geese, chicken, turkey, Japanese quail, Indian peafowl, and ring-necked pheasant, respectively. Also, the sequence comparisons results of Egyptian duck breeds *PRL* gene exon 5 and the other avian species revealed that Moulard allele A, Campbell allele A, and Muscovy allele B) shared similarity (97.75%, 97.50%, 83.89%, 84.48%, 83.54%, 87.17% and 84.20%) and (Moulard allele B , Campbell allele B, Muscovy allele A and Pekin) shared similarity (100.00%, 98.75%, 85.68%, 85.75%, 85.09%, 87.96% and 86.65) with *A. platyrhynchos* (Moulard duck), goose, chicken, turkey, ring-necked pheasant, Ostrich and Indian peafowl, respectively.

The PRL gene has been cloned formerly in divergent avian species like pigeon, duck, chicken, quail, turkey and pigeon (Liu et al. 2008). Duck *PRL* was found to have sequence identity (92.0%, 91.7% and 91.4%) at the cDNA level compared to *PRL* of chicken, turkey and quail, respectively. The mature duck *PRL* has an overall similarity with a comparable region of chicken (95.5%), turkey (92.5%) and quail (95.5%) *PRL* (Kansaku et al., 2005). Also, the sequence analysis of the proximal region, of duck PRL promoter, displayed a high plane of similarity to turkey and chicken PRL promoter. These results viewed that the mechanisms, modulating the gene expression, may be vastly conserved in avian species (Kansaku et al., 2005). Over and above, since the avian PRL gene was cloned and sequenced, most researches illuminate new polymorphic sites in this gene (Rashidi et al., 2012).

The phylogenetic tree of exons 1 and 5 for DNA *PRL* gene in the different avian species showed that the locus of four Egyptian duck was related to *PRL* gene of *A. platyrhynchos*, *A. anser* more than of *G. gallus*, *M. gallopavo*, *P. colchicus*, *S. camelus* and *P. cristatus*. This clustering based on both of nucleotide of *PRL* gene clearly showed that the phylogenetic inter-relationship among these species and is generally in agreement with the known species relationships. This tree was constructed using Mr. Bayes, employing a previously calculated gene family with multiple sequence alignment (Huelsenbeck and Ronquist, 2001; Siltberg and Liberles, 2002).

CONCLUSION

The results of this study manipulating the four Egyptian duck breeds; Campbell, Moulard, Muscovy and Pekin, supported previous findings. That there are many SNPs found in the sequences in the four Egyptian duck breeds. The utmost vital ones, are those found at the restriction sites of *Xba*I, for the amplified fragment contained the promotor, exon 1 and intron 1 (T378C), and exon 5/*Dra*I (A5871G). These data could serve as a basis for further insight into this avian gene. Owing to the fact that the genotypes distribution in this studied between the four Egyptian duck breeds was not in Hardy–Weinberg equilibrium, to confirm the observed associations, further research should be applied to a broad and more homogeneous population of ducks. In addition, a survival evaluation and post slaughter examination of ducks must be carried out to ascertain any potential connections with polymorphic variants in the PRL gene present in duck.

DECLARATIONS

Acknowledgements

The authors sincere gratefulness to the Nubaria experimental farm, for kind assistance in collecting the blood samples of the duck breeds understudy, needed for accomplishment of this research.

Authors' contributions

All authors contributed evenly throughout the manuscript. All authors participated equally in the study plan and design. EME, NMS, DMM and MAM collected the samples from different locations and isolated the nucleic acids. NMS, DMM and MAM carried out PCR and sequencing analyses. NMS, DMM, MAM and KFM carried out the statistical analysis of data and reported the results of the molecular analysis. KFM, NMS and MAM collaborated on writing, revising, and improvement of the article for publication. All authors read and approved the final.

Competing interests

The authors declare that they don't have any conflict of interest.

REFERENCES

Alsiddig MA, Yu SG, Pan ZX, Widaa H, Badri TM, Chen J, and Liu HL (2017). Association of single nucleotide polymorphism in melatonin receptor 1A gene with egg production traits Journal in Yangzhou geese. Animal Genetics, 48: 245–249. DOI: https://www.doi.org/ 10.1111/age.12517

Asiamah AC, Zou, K, Lu LL, Zhang SW, Xue Y, Su Y, and Zhao Z (2019). Genetic effects of polymorphisms of candidate genes associated with ovary development and egg production traits in ducks. Animal Reproduction Science, 11: 106-219. DOI: https://www.doi.org/10.1016/j.anireprosci. 2019.106219

Baeza E (2006). Effects of genotype, age and nutrition on intramuscular lipids and meat quality. In Symposium COA/INRA Scientific Cooperation in Agriculture. November, pp. 7-10. Available at: http://www.angrin.tlri.gov.tw/INRA/o5.pdf

Bai DP, Hu YQ, Li YB, Huang ZB, and Li A (2019). Polymorphisms of the prolactin gene and their association with egg production traits in two Chinese domestic ducks. British poultry science, 60(2): 125-129. DOI: https://www.doi.org/10.1080/00071668.2019.1567909

Basumatary K, Das B, Borah P, Barkalita L, Bharali K, and Tamuly S (2019). Polymorphism of prolactin receptor gene in indigenous ducks of Assam. Journal of Entomology and Zoology Studies, 7(1): 922-925. Available at: http://www.entomoljournal.com/archives/2019/vol7issue1/PartO/7-1-143-769

Chang MT, Cheng YS, and Huang MC (2012). Association of prolactin haplotypes with reproductive traits in Tsaiya ducks. Animal reproduction science, 135(1-4):91-96. DOI: https://www.doi.org/10.1016/j.anireprosci.2012.08.024

Chuekwon K, and Boonlum S (2017). Association of Prolactin Gene with Egg Production in Khaki Campbell Ducks. Walailak Journal of Science and Technology, 14(11): 849–853. Available at: http://wjst.wu.ac.th/index.php/wjst/article/download/1934/723/29029

Cui JX, Du HL, Liang Y, Deng XM, Li N, and Zhang XQ (2006). Association of polymorphisms in the promoter region of chicken prolactin with egg production. Poultry Science, 85(1):26-31. DOI: https://www.doi.org/10.1093/ps/85.1.26

Elsholtz HP, Lew AM, Albert PR, and Sundmark VC (1991). Inhibitory control of prolactin and Pit-1 gene promoters by dopamine. Dual signaling pathways required for D2 receptor-regulated expression of the prolactin gene. Journal of Biological Chemistry, 266(34): 22919-22925. Available at: https://www.jbc.org/content/266/34/22919.short

Feng P, Zhao W, Xie Q, Zeng T, Lu L, and Yang L (2018). Polymorphisms of melatonin receptor genes and their associations with egg production traits in Shaoxing duck. Asian-Australasian Journal of Animal Science, 31(10): 1535–1541. DOI: https://www.doi.org/10.5713/ajas.17.0828

Huelsenbeck JP, and Ronquist F (2001). Mr. Bayes: Bayesian inference of phylogeny. Bioinformatics, 17: 754–755. DOI:https://doi.org/10.1093/bioinformatics/17.8.754

Jiang RS, Zhang LL, Geng ZY, Yang T, and Zhang SS (2009). Single nucleotide polymorphisms in the 5'-flanking region of the prolactin gene and the association with reproduction traits in geese. South African Journal of Animal Science, 39: 83-87. DOI: https://www.doi.org/10.4314/sajas.v39i1.43550

Kang L, Zhang N, Zhang Y, Yan H, Tang H, Yang C, Wang H, and Jiang Y (2012). Molecular characterization and identification of a novel polymorphism of 200 bp indel associated with age at first egg of the promoter region in chicken follicle-stimulating hormone receptor (FSHR) gene. Molecular Biology Reports, 39: 2967–2973. DOI: https://www.doi.org/10.1007/s11033-011-1058-x

Kansaku N, Hiyama G, Sasanami T, and Zadworny D (2008). Prolactin and growth hormone in birds: Protein structure, gene structure and genetic variation. The Journal of Poultry Science, 45(1): 1-6. DOI: https://www.doi.org/10.2141/jpsa.45.1

Kansaku N, Ohkubo T, Okabayashi H, Guémené D, Kuhnlein U, Zadworny D, and Shimada K (2005). Cloning of duck PRL cDNA and genomic DNA. General and Comparative Endocrinology, 141(1): 39-47. DOI: https://www.doi.org/10.1016/j.ygcen.2004.11.017

Kulibaba RA (2015). Polymorphism of Growth Hormone, Growth Hormone Receptor, Prolactin and Prolactin Receptor genes in connection with egg production in Poltava clay chicken. Sel'Skokhozyaistvennaya Biologia, 50(2): 198-207. DOI: https://www.doi.org/10.15389/agrobiology.2015.2.198eng

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, and Lopez R (2007). Clustal W and Clustal X version 2.0. Bioinformatics, 23(21): 2947–2948. DOI: https://www.doi.org/10.1093/bioinformatics/btm404

Li HF, Zhu WQ, Chen KW, Zhang TJ, and Song WT (2009). Association of polymorphisms in the intron 1 of duck prolactin with egg performance. Turkish Journal of Veterinary and Animal Sciences, 33 (3): 193-197. DOI: https://www.doi.org/10.3906/vet-0709-4

Li J, Liang A, Li Z, Du C, Hua G, Salzano A, Campanile G, Gasparrini B, and Yang L (2017). An association analysis between *PRL* genotype and milk production traits in Italian Mediterranean river buffalo. Journal of Dairy Research, 84: 430-433. DOI: https://www.doi.org/10.1017/s0022029917000693

Liu HG, Wang XH, Liu YF, Zhao XB, Li N, and Wu CX (2007). Analysis of the relationship between codon frequency of prolactin gene and laying performance in five chicken breeds. Progress in Biochemistry and Biophysics, 34: 1101-1106. Available at: http://www.pibb.ac.cn/

Liu Z, Shiz D, Liu Y, Lim Y, Huangy M, and Yao BH (2008). Molecular cloning and characterization of the Magang goose prolactin gene. General and Comparative Endocrinology, 155: 208-216. DOI: https://www.doi.org/10.1016/j.ygcen.2007.04.017

Mazurowski A, Frieske A, Wilkanowska A, Kokoszyński D, Mroczkowski S, Bernacki Z, and Maiorano G (2016). Polymorphism of prolactin gene and its association with growth and some biometrical traits in ducks. Italian Journal of Animal Science, 15: 200-206. DOI: https://www.doi.org/10.1080/1828051X.2016.1153405

Mohamed MMO, Shaaban AH, Hassanin AAI, and Hussein WA (2017). Polymorphism of prolactin gene and its association with egg

production trait in four commercial chicken lines. Journal of the Hellenic Veterinary Medical Society, 68: 391–404. DOI: https://www.doi.org/10.12681/jhvms.15502

Narhari D (2009). Housing and management of ducks. IV World Waterfowl Conference, 11-13 November, 2009, Thrissur, India, pp. 45-47. Available at: http://www.waterfowl2009.vetcos.com/proceedings

Rashidi H, Rahimi-Mianji G, Farhadi A, and Gholizadeh M (2012). Association of prolactin and prolactin receptor gene polymorphisms with economic traits in breeder hens of indigenous chickens of Mazandaran province. Iranian journal of Biotechnology, 10(2): 129-135. Available at: http://www.ijbiotech.com/

Sadar MJ, Guzman DSM, Burton AG, Byrne BA, Wiggans KT, and Hollingsworth SR (2014). Mycotic keratitis in a khaki campbell duck (*Anas platyrhynchos domesticus*). Journal of avian medicine and surgery, 28(4): 322-329. DOI: https://www.doi.org/10.1647/2013-050

Sarvestani BAS, Niazi A, Zamiri MJ, and Taromsari DM (2013). Polymorphisms of prolactin gene in a native chicken population and its association with egg production. Iranian Journal of Veterinary Research, 14: 113-119. DOI: https://www.doi.org/10.22099/IJVR.2013.1584

Shamsalddini S, Mohammadabadi MR, and Esmailizadeh AK (2016). Polymorphism of the prolactin gene and its effect on fiber traits in goat. Genetika, 52(4): 461–465. DOI: https://www.doi.org/10.7868/S0016675816040093

Shokrzadeh M, and Mohammadpour A (2018). Evaluation of a modified salt-out method for DNA extraction from whole blood lymphocytes: A simple and economical method for gene polymorphism. Pharmaceutical and Biomedical Research, 4(2). DOI: https://www.doi.org/10.18502/pbr.v4i2.218

Siltberg J, and Liberles DA (2002). A simple covarion-based approach to analyze nucleotide substitution rates. Journal of Evolutionary Biology, 15(4): 588-594. DOI: https://www.doi.org/10.1046/j.14209101.2002.00416.x

Tempfli K, Konrád S, KovácsnéGaál K, Pongrácz L, and Papp BÁ (2015). Prolactin, dopamine receptor D1 and Spot14α polymorphisms affect production traits of Hungarian Yellow hens. Livestock Science, p. 174. DOI: https://www.doi.org/10.1016/j.livsci.2015.01.012

Veeramani P, Prabakaran R, Sivaselvam SN, Sivakumar T, Selvan ST, and Karthickeyan SMK (2016). Phylogenetic analysis of six duck populations. Indian Journal of Animal Research, 50(4): 626-628. DOI: https://www.doi.org/10. 18805/ijar.9301

Wang C, Liang Z, Yu W, Feng Y, Peng X, Gong Y, and Li S (2011). Polymorphism of the prolactin gene and its association with egg production traits in native Chinese ducks. South African Journal of Animal Science,41:63-69. Available at: http://www.sasas.co.za

Watahiki M, Tanaka M, Masudam N, Sugisakim K, Yamamatom M, Yamakawa M, Nagai J, and Nakashima K (1989). Primary structure of chicken pituitary prolactin deduced from the cDNA sequence. Conserved and specific amino acid residues in the domains of the prolactins. The Journal of Biological Chemistry, 264: 5535-5539. Available at: https://www.jbc.org/content/264/10/5535.long

Wawro K, Wilkiewicz-Wawro E, Kleczek K, and Brzozowski W (2004). Slaughter value and meat quality of Muscovy ducks, Pekin ducks and their crossbreeds. Archiv fur Tierzucht, 47(3): 287-299. DOI: https://www.doi.org/10.5194/aab-47-287-2004

Xu ZQ, He J, Ji CL, Zhang Y, Nie QH, Zhang DX, and Zhang XQ (2015). Polymorphisms in the 5'-flanking regions of the GH, PRL, and Pit-1 genes with Muscovy duck egg production. Journal of Animal Science, 93: 28-34. DOI: https://www.doi.org/10.2527/jas.2014-8071

Yadav SK, Maurya SK, Yadav AK, Kumar A, and Yadav K (2018). Study of prolactin receptor gene (PRLR5) polymorphisms and its association with egg production in Kadaknath hens. Indian Journal of Animal Research, 52(8): 1232-1235. DOI: https://www.doi.org/10.18805/ijar.v0iOF.8001

Yurnalis A, Kamsa Z, and Putra DE (2019). Polymorphism of prolactine genes and its association with body weight in Bayang ducks, local duck from West Sumatera, Indonesia. International Conference on Animal Production for Food Sustainability. IOP Conf. Series: Earth and Environmental Science, 287: 012009. DOI: https://www.doi.org/10.1088/17551315/287/1/02009

Zeng T, Chen L, Du X, Lai SJ, Huang SP, Liu YL, and Lu LZ (2016). Association analysis between feed efficiency studies and expression of hypothalamic neuropeptide genes in laying ducks. Animal genetics, 47(5): 606-609. DOI: https://www.doi.org/10.1111/age.12457

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication
J. World Poult. Res. 10(4): 599-604, December 25, 2020
Research Paper, PII: S2322455X2000068-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.68

The Effect of Anchovy Fish Supplementation on the Level of N-3 LC-PUFA in Egg Yolk

Marcelia Sugata[1*], Amanda Atmadja[1], Andrew Darmawan[1], Yehezkiel Tatulus[1], Stefanie C. Djojo[1], Denny Rizkinata[1], Dela Rosa[2], Hans Victor[1] and Tan T. Jan[1]

[1]*Department of Biology, Universitas Pelita Harapan, Jl. M.H. Thamrin Boulevard, Tangerang 15811, Indonesia*
[2]*Department of Pharmacy, Universitas Pelita Harapan, Jl. M.H. Thamrin Boulevard, Tangerang 15811, Indonesia*

*Corresponding author's Email: marcelia.sugata@uph.edu; ORCID: https://orcid.org/0000-0002-2879-6990

Received: 25 Oct. 2020
Accepted: 09 Dec. 2020

ABSTRACT

Since the recommended daily intake of n-3 LC-PUFA is rarely met, interest in food enrichment has been increasing. It is known that dietary supplementation could alter the level and type of PUFA in the egg. Hence, the present study focused on the enrichment of egg yolk by the addition of 10% anchovy fish to the chicken diet. Based on gas chromatography analysis, dried and pre-dried anchovy from Indonesia contained a considerable amount of total eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which was 60.10 g and 68.80 mg/100 g, respectively. After 24 days of anchovy supplementation, DHA-rich anchovy fish oil diet caused a significant increase of DHA but not EPA in egg yolk. Hens fed with anchovy could produce eggs with a higher amount of total EPA and DHA, which was up to 155.98-201.53%, as compared to control eggs. Furthermore, the sensory profile of control and enriched eggs was also evaluated. There was no significant difference in texture, aroma, flavor, and appearance between control and enriched eggs. In conclusion, this study indicated that anchovy fish supplementation could increase the level of EPA and DHA in egg yolk without causing any sensory changes in the yolk.

Keywords: Anchovy, DHA, Egg yolk, Enrichment, EPA

INTRODUCTION

Recent nutritional trends show that interest in enriched food products with omega-3 polyunsaturated fatty acids (n-3 PUFA) has been increasing. Due to their biological effects, long chain (LC) n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have gained more attention than the shorter chain n-3 PUFA α-linolenic acid (ALA). Adequate consumption of EPA and DHA has been proven to provide various health benefits, including the promotion of visual and neural development in fetus and young children, the reduction of cardiovascular disease and the attenuation of inflammation and some cancers (Lemahieu et al., 2015). Although proven to be beneficial for health, the amount of EPA and DHA obtained from the conversion of ALA in human body is not efficient. Hence, human needs to obtain n-3 LC-PUFA through their diet (Komprada, 2012).

Fatty fishes and chicken eggs are known to be rich in n-3 LC-PUFA. Atlantic salmon, which is known as gold standard for omega-3 fatty acid, contains 0.321% EPA and 1.115% DHA (FDC ID: 173686, USDA). Meanwhile, European raw anchovy contains 0.538% EPA and 0.911% DHA (FDC ID: 174182, USDA) (USDA, 2020). Even with the low-fat content, white fish such as milkfish was reported to have 0.36% EPA and 1.17% DHA (Sugata et al., 2019). Although fishes are rich in n-3 LC-PUFA, they are not consumed as much as eggs. Eggs are usually consumed as food or used as food ingredient. Thus, as compared to the fishes, eggs enriched with n-3 LC-PUFA could be one of the best options to fulfil the recommended daily intake of n-3 LC-PUFA.

Each chicken egg commonly contains 1.5 mg of EPA and 47.5 – 51.0 mg of DHA (Kovalcuks, 2014). Based on Food Data Central (USDA), every 100 g of egg yolk contains 11 mg EPA and 114 mg DHA (FDC ID: 783929). However, various feeding strategies have been applied to increase the level of PUFA in eggs. Egg yolk composition, such as EPA and DHA content, is closely

To cite this paper: Sugata M, Atmadja A, Darmawan A, Tatulus Y, Djojo SC, Rizkinata D, Rosa D, Victor H and Jan TT (2020). The Effect of Anchovy Fish Supplementation on the Level of N-3 LC-PUFA in Egg Yolk. *J. World Poult. Res.*, 10 (4): 599-604. DOI: https://dx.doi.org/10.36380/jwpr.2020.68

related to the lipids consumed by the laying hens. Enrichment of eggs with different sources of n-3 PUFA such as DHA-rich microalgae (Ao et al., 2015), ALA-rich flaxseed (Ehr et al., 2017), and DHA-rich fish oil (Lawlor et al., 2010) has been widely investigated. Previous study had been reported that ALA in plants, such as flaxseed, was effective to increase linolenic acid (LNA, 18:3 n-3) in eggs, but not EPA and DHA (Ehr et al., 2017). Meanwhile, fish oil could enrich both EPA and DHA levels in eggs (Ao et al., 2015). However, Bruneel et al. (2013) found that enrichment of eggs with n-3 PUFA could increase the level of DHA, but it was not effective on EPA level.

The most of n-3 PUFA sources used in previous studies had relatively expensive prices and were difficult to obtain in Indonesia, hence, the use of those ingredients for this study is not feasible. Indonesia is an agricultural country with high amount of fish production. In 2017, the production of anchovy fish, which is known as "ikan teri", in Indonesia reached about 3% of the world production of anchovy (MMAFRI, 2018). Moreover, to the best of authors' knowledge, enrichment of egg yolk with anchovy fish as n-3 PUFA source has not been widely studied. Therefore, this research aimed to investigate the effect of anchovy fish supplementation on the level of n-3 LC-PUFA in egg yolk.

MATERIALS AND METHODS

Source of n-3 Polyunsaturated Fatty Acids

Indonesian anchovy used in this study was *Stolephorus* sp. (based on certification from Indonesian Institute of Sciences). There were two kinds of anchovy obtained from the market: dried and fresh. Dried anchovy was fresh anchovy that had been dried before reached the market, hereinafter referred to as dried anchovy. Meanwhile, fresh anchovy was raw anchovy that was available in the market without going through the drying process. Prior to experiment, fresh anchovy was sun-dried, hereinafter referred to as pre-dried anchovy.

Lipid extraction from anchovy fish

To analyze n-3 PUFA levels, the lipid fraction of anchovy was extracted according to Bligh and Dyer (1959). One hundred gram of dried anchovy was homogenized with chloroform/methanol/water (2:2:1.8, v/v/v) as solvents. The mixture was then filtrated and centrifuged at 1000 rpm for 10 min. The lipid fraction was collected from chloroform layer at the bottom and then filtered. Afterwards, the solvents were evaporated at 50°C with agitation at 120 rpm. The lipid fraction must be converted to methyl esters (FAME) prior to analysis using gas chromatography (Harynuk et al., 2006). Fifteen milligrams of fish oil were methylated with one ml of 14% BF3-MeOH in boiling water for 7 min. After cooling, hexane (1 ml) and water were added to the mixture, followed by vigorous shaking. Phase layers would be formed, and FAMEs can be found in hexane layer on the top.

Animals' diets and egg collection

Prior to experiment, nine laying hens (28 weeks of age, Hisex Brown) from Rizky Farm (Bogor, Indonesia) were fed with commercial chicken feed. The laying hens were placed individually in cage without any environmental control and divided into three groups of three hens. The control group continued to receive 120 g commercial feed per day, while the treated groups received the commercial feed supplemented with anchovy fish (10% w/w). In other word, treated group received a mixture of 12 g anchovy fish and 108 g commercial feed every day (total feed: 120 g). The group of hens fed with dried anchovy hereinafter referred to as treatment group A, while another group fed with pre-dried anchovy hereinafter referred to as treatment group B. Feed and water were given ad libitum. For each group, laid eggs were collected at either day 20-21 (almost the end of supplementation period) and at either day 23-24 (end of supplementation period). All collected eggs were stored at 4°C until analysis.

Lipid extraction from chicken egg yolk

The extraction of lipid fraction from egg yolk was carried out according to Kovalcuks (2014). One part of egg yolk was added with two part of the mixture of hexane and isopropanol (70:30). The mixture was homogenized using a magnetic stirrer for 30 minutes at room temperature and then filtered using Whatmann filter paper number 1. The solvent was evaporated using a rotary evaporator at 80°C and a speed of 30 rpm. The lipid fraction was then methylated prior to analysis using gas chromatography. A modified method by Morrison and Smith (1964) was used to form methyl esters fatty acid (FAME) from egg yolk lipid. Twenty-five microliters of lipid were methylated with 2 ml of BF3-MeOH 14% at 60°C for 10 minutes. Water (1 ml) was added to the solution, and then FAMEs were extracted with hexane (1 ml). FAMEs were then dried over sulfate anhydrous.

N-3 Polyunsaturated Fatty Acids in anchovy and egg yolk

FAMEs were analyzed by gas chromatography (Agilent 7890A Series GC System) equipped with a 5975C Mass Selective Detector. The separation was performed on a fused-silica capillary column (0.25 µm; 30 m x 0.25 mm i.d.; DB-wax; Agilent Technologies, USA). The flow rate of the helium carrier gas was 2,69 ml/ min. The injection was operated in pulsed splitless mode at a temperature of 260°C, while the detector was set at 280°C. For analysis, one microliter of FAME was automatically injected (Agilent 7693, Agilent Technologies, USA). The column oven was set to maintain a temperature of 50°C for 1 min, then risen 25°C/ min to 200°C, and 2°C/ min to 250°C, followed by a plateau of 250°C for 20 min. The mass spectrometer was operated in selected ion monitoring (SIM) mode.

Preparation of Sensory evaluation

Thirty volunteers were involved in consumer acceptance test to assess the difference between control and enriched eggs after hard-boiled. Eggs were placed in boiling water for 10 min. After cooled, shells were removed, and each egg cut into quarters. No salt was used. There were four categories for the assessment, including appearance, texture, flavor, and aroma, which scored based on hedonic scale (1-extremely disliked; 6- extremely liked). Appearance was specified to the color of egg yolk, assuming more lipid content would give thicker yolk color, which was considered as more attractive. Texture was defined as the overall measure of oral sensations associated with placing food in the mouth. Flavor was emphasized at the savory degree of the egg, assuming higher oil content would give tastier flavor. Aroma was referred to the presence of "fishy" odor, meaning less preference in aroma indicates more "fishy" odor from the sample.

Sensory evaluation methodology

Each panelist was given two hard-boiled eggs, the first one was the control egg and the other one was the enriched egg. Both control and enriched egg were blind-coded. Each panelist was asked to evaluate these blind-coded eggs by giving a hedonic scale. Assessment was calculated by the following: Σ (number of responses × hedonic scale) / total panelist, with total number of all responses = total panelist.

Statistical analysis

The results were statistically analyzed using Minitab 15 (Minitab Inc., USA) software. All data were stated as the mean ± Standard Deviation (SD) with statistical significance at $p < 0.05$.

RESULTS AND DISCUSSION

The productive age of laying hens ranges from 28 to 97 weeks and the weight of egg yolk produced could increase with the age of hens. In general, the weight of egg yolk increases proportionally to its total lipid content (Ahn et al., 1997). However, Lawlor et al. (2010) reported that the addition of microencapsulated fish oil (20 g/kg) in chicken diet could decrease the weight of egg yolk by 5.29%. Since the consumption of n-3 LC-PUFA might decrease serum triglycerides in the hens, less lipids are available for yolk formation (Fraeye et al., 2012).

Dried anchovy contained approximately 3.33–3.59% lipid, meaning there was 0.40–0.43 g lipid in 12 g anchovy (10% w/w supplementation). Thus, the percentage of additional lipid for treatment groups was approximately 0.33–0.36% (in 120 g chicken feed). According to Khosravinia et al. (2014), adding too much fish oils to chicken feed could increase the concentration of urea and uric acid in chickens. Through the addition of 2% fish oil, Lawlor et al. (2010) reported that DHA content in chicken eggs could be increased by 54.48%. Meanwhile, Lemahieu et al. (2015) showed that supplementation of 0.68 % fish oil resulted in enrichment efficiency of n-3 LC-PUFA of more than 55%.

The results of this study showed that anchovy contained more DHA than EPA. According to Codabaccus et al. (2012), fatty fishes tend to use dietary EPA for β-oxidation, but not DHA. This might explain why EPA was not present in excess, while DHA can be found at a relatively high level in anchovy. The level of total EPA and DHA in dried and pre-dried anchovy was 60.10 and 68.80 mg/100 g, respectively. Dried anchovy was already in dried form when it was on the market; hence, the fish might have been stored for some time. On the other hand, pre-dried anchovy was still fresh when it was on the market and was dried immediately without long storage period. Crypian et al. (2017) reported that the level of PUFAs could decrease during storage because PUFAs are highly susceptible to oxidation. This might also the case with dried anchovy.

After anchovy supplementation, n-3 LC-PUFA in egg yolks was examined using GC-MS. Table 1 shows that daily enrichment with 0.33–0.36% anchovy fish oil increased the level of EPA and DHA in egg yolk, but only DHA content increased significantly ($p < 0.05$). In the end of supplementation period, the addition of dried and pre-dried anchovy increased EPA and DHA in egg yolk by 155.98% and 201.53%, respectively. Since total EPA and DHA content in pre-dried anchovy was higher than dried anchovy, it was expected that treatment group B produced eggs with higher increase of total EPA and DHA as compared to treatment group A. Other studies reported that the addition of 2% fish oil in chicken diet could increase the DHA content in eggs by 64% (Lawlor et al., 2010) or up to 300% (Gonzalez-Esquerra and Leeson, 2001). In accordance with Lawlor et al. (2010), EPA-rich and DHA-rich fish oil diet caused significant increase ($p < 0.05$) of DHA, but not EPA, in egg yolk. Since most EPA is converted to DHA before it is deposited, the major n-3 LC-PUFA in eggs is DHA (Cachaldora et al., 2008).

Table 1. The increase of EPA and DHA in the chicken eggs at the start (day 1) and at the end (day 23-24) of supplementation period

Day	Description	Yolk weight (g/egg)	Total lipid (g/egg)	Increase of DHA content per egg (%)	Increase of EPA content per egg (%)	Increase of total EPA+DHA (%)
0	Control	14.05 ± 0.59[a]	2.15 ± 0.43	-	-	-
20-21	Treatment group A	15.63 ± 0.87[b]	2.57 ± 0.39	70.08 ± 5.28[a]	6.31 ± 3.30[a]	65.89 ± 5.15[a]
	Treatment group B	15.43 ± 1.00[a]	2.60 ± 0.50	162.31 ± 8.05[b]	65.92 ± 7.09[b]	155.98 ± 7.85[b]
23-24	Treatment group A	14.79 ± 0.19[a]	2.18 ± 0.11	201.76 ± 7.71[c]	113.29 ± 22.53[c]	195.90 ± 7.56[c]
	Treatment group B	14.47 ± 0.32[a]	2.14 ± 0.52	213.27 ± 6.90[c]	34.55 ± 5.63[d]	201.53 ± 6.64[c]

Note: The table shows the data from eggs produced by hens fed with commercial feed (control), commercial feed supplemented with 10% dried anchovy (treatment group A), and commercial feed supplemented with 10% pre-dried anchovy (treatment group B). Day indicates the period of anchovy supplementation. Values with the different letter in the same column are significantly different ($p < 0.05$).

	Control	Treatment
Texture	4.63	4.63
Aroma	4.70	4.77
Appearance	4.80	4.90
Flavor	4.83	5.00

Figure 1. The overall assessment of control (— —) and enriched eggs (— —)

Although enrichment period showed a good correlation with the increase of n-3 LC-PUFA in egg yolk, in this study, the supplementation was limited for 24 days. Further studies need to be done to investigate the effect of longer enrichment period on the increase of EPA and DHA content in egg yolk, or even in chicken meat. Since about 60% of egg production costs comes from chicken feed, the addition of 10% anchovy will increase the feed cost. However, the price of omega-3 enriched eggs on the market is also double as compared to bulk eggs. Despite the more expensive price, modern society has high interest in food enrichment with n-3 PUFA due to its health benefits. Thus, the use of anchovy is expected to give little or no burden on chicken farmers.

N-3 LC-PUFA can be oxidized easily and give undesirable off-flavors in food products. Since fish oil supplementation could increase the level of lipid in egg yolk, higher extent of lipid oxidation might occur (Fraeye et al., 2012). The amount of lipid in the eggs is strongly correlated with the "fishy" taste and odor, hence, the amount of fish used for feeding strategies needs to be confined. Gonzalez-Esquerra and Leeson (2001) found that western consumers could not accept eggs produced

from inclusion of more than 1.5% of fish oil. In this study, to analyze the acceptance of panelist to the eggs enriched with 0.33–0.36% anchovy fish oil, sensory evaluation was done based on organoleptic profile of control and enriched eggs (Figure 1). Overall assessment on texture, aroma, flavor and appearance of both control and enriched eggs indicated that even though the panelist might not be able to distinguish between control and enriched eggs ($p \geq 0.05$), the enriched eggs were more preferred.

CONCLUSION

This study showed that the level of n-3 polyunsaturated fatty acids in egg yolk could be increased by the supplementation of anchovy fish in chicken diet. The supplementation of docosahexaenoic acid (DHA)-rich anchovy fish for 24 days in chicken diet caused significant increase of docosahexaenoic acid, but it was not effective on eicosapentaenoic acid in egg yolk. Furthermore, the evaluation of sensory profile of control and enriched eggs showed that there was no significant difference on texture, aroma, flavor and appearance between control and enriched eggs.

DECLARATIONS

Acknowledgements

This work was supported financially by the Centre for Research and Community Development (LPPM), Universitas Pelita Harapan (P-025-FaST-I-2019). The authors would like to thank the Biology Department of Universitas Pelita Harapan for allowing us to conduct this research in the Advanced (407) and Fundamental Biology Laboratory (202). The authors also acknowledge with much appreciation the support given by Rizky Farm.

Competing interests

The authors have declared that no competing interest exists.

Authors' contributions

TTJ formulated the concepts. AA, AD, YT and SCD collected the data and drafted the manuscript. DR[1] supported data collection. MS, HV and DR[2] analyzed the data and prepared the figures, tables. MS finalized the manuscript.

REFERENCES

Ahn DU, Kim SM, and Shu H (1997). Effect of egg size and strain and age of hens on the solids content of chicken eggs. Poultry Science Journal, 76: 914-919. DOI: https://www.doi.org/10.1093/ps/76.6.914

Ao T, Macalintal LM, Paul MA, Pescatore AJ, Cantor AH, Ford MJ, Timmons B, and Dawson KA (2015). Effects of supplementing microalgae in laying hen diets on productive performance, fatty-acid profile, and oxidative stability of eggs. Journal of Applied Poultry Research, 24: 394-400. DOI: https://www.doi.org/10.3382/japr/pfv042

Bligh EG, and Dyer WJ (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37: 911- 917. DOI: https://www.doi.org/10.1139/y59-099

Bruneel C, Lemahieu C, Fraeye I, Ryckebosch E, Muylaert K, Buyse J, and Foubert I (2013). Impact of microalgal feed supplementation on omega-3 fatty acid enrichment of hen eggs. Journal of Functional Foods, 5: 897-904. DOI: https://www.doi.org/10.1016/j.jff.2013.01.039

Cachaldora P, Garcia-Rebollar P, Alvarez C, De Blas JC and Mendez J (2008). Effect of type and level of basal fat and level of fish oil supplementation on yolk fat composition and n−3 fatty acids deposition efficiency in laying hens. Animal Feed Science and Technology, 141: 104–114. DOI: https://www.doi.org/10.1016/j.anifeedsci.2007.05.024

Codabaccus VM, Carter CG, Bridle AR, and Nichols PD (2012). The "n−3 LC-PUFA sparing effect" of modified dietary n−3 LC-PUFA content and DHA to EPA ratio in Atlantic salmon smolt. Aquaculture, 356-357: 135-140. DOI: https://www.doi.org/10.1016/j.aquaculture.2012.05.024

Crypia OO, Sveinsdottir K, Nguyen MV, Tomasson T, Thorkelsson G and Aarason S (2017). Influence of lipid content and packaging methods on the quality of dried capelin (Mallotus villosus) during storage. Journal of Food Science and Technology, 54(2): 293–302. DOI: https://www.doi.org/10.1007/s13197-016-2462-y

Ehr IJ, Persia ME and Bobeck EA (2017). Comparative omega-3 fatty acid enrichment of egg yolks from first-cycle laying hens fed flaxseed oil or ground flaxseed. Poultry Science, 96: 1791-1799. DOI: https://www.doi.org/10.3382/ps/pew462

Fraeye I, Bruneel C, Lemahieu C, Buyse J, Muylaert K and Foubert I (2012). Dietary enrichment of eggs with omega-3 fatty acids: A review. Food Research International, 48: 961-969. DOI: https://www.doi.org/10.1016/j.foodres.2012.03.01

Gonzàlez-Esquerra R and Leeson S (2001). Alternatives for enrichment of eggs and chicken meat with omega-3 fatty acids. Canadian Journal of Animal Science, 81: 295-305. DOI: https://www.doi.org/10.4141/a00-092

Harynuk J, Wynne PM and Marriott PJ (2006). Evaluation of new stationary phase for the separation of fatty acid methyl esters. Chromatographia, 63: 61-66. DOI: https://www.doi.org/10.1365/s10337-006-0714-0

Khosravinia H, Azarfar A and Sokhtehzary A (2014). Effects of substituting fish meal with poultry by-product meal in broiler diets on blood urea and uric acid concentrations and nitrogen content of litter. Journal of Applied Animal Research, 43: 191-195. DOI: https://www.doi.org/10.1080/09712119.2014.963085

Komprada T (2012). Eicosapentaenoic and docosahexaenoic acids as inflammation-modulating and lipid homeostasis influencing nutraceuticals: A review. Journal of Functional Foods, 4: 25–38. DOI: https://www.doi.org/10.1016/j.jff.2011.10.008

Kovalcuks A (2014). Solvent extraction of egg oil from liquid egg yolk. Annual 20th ISC Research for Rural Development, Latvia 1: 142-147.

Lawlor JB, Gaudette N, Dickson T and House JD (2010). Fatty acid profile and sensory characteristics of table eggs from laying hens fed diets containing microencapsulated fish oil. Animal Feed Science and Technology,156:97–103.DOI: https://www.doi.org/10.1016/j.anifeedsci.2010.01.003

Lemahieu C, Bruneel C, Ryckebosch E, Muylaert K, Buyse J and Fouber I (2015). Impact of different omega-3 polyunsaturated fatty acid (n-3 PUFA) sources (flaxseed, *Isochrysis galbana*, fish oil and DHA Gold) on n-3 LC-PUFA enrichment (efficiency) in the egg yolk. Journal of Functional Foods, 19: 821-827. DOI: https://www.doi.org/10.1016/j.jff.2015.04.021

Ministry of Maritime Affairs and Fisheries of the Republic of Indonesia (MMAFRI) (2019). Indonesian Fisheries Productivity. Available from https://kkp.go.id/wp-content/uploads/2018/01/KKP-Dirjen-PDSPKP-FMB-Kominfo-19-Januari-2018.pdf. Accessed May 15, 2019.

Morrison WR and Smith LM (1964). Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. Journal of Lipid Research, 5: 600-608.

Sugata M, Wiriadi PF, Lucy J and Tan TJ (2019). Total lipid and omega-3 content in Pangasius catfish (Pangasius pangasius) and milkfish (Chanos chanos) from Indonesia. Malaysian Journal of Nutrition, 25: 163-169. DOI: https://www.doi.org/10.31246/mjn-2018-013

U.S. Department of Agriculture (USDA). Food Data Central (2020). Available at: https://fdc.nal.usda.gov/index.html. Accessed May 1, 2020.

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 605-614, December 25, 2020

Research Paper, PII: S2322455X2000069-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.69

Molecular Breeding of Three Genes Associated with Egg Production Traits in Three Strains of Chickens

Waleed S. El-Tahawy[1] and Manal M. Abdel-Rahman[2*]

[1]*Animal and Poultry Production Department, Agriculture Faculty, Damanhour University, Egypt*
[2]*Genetic Engineering and Biotechnology Laboratory, Plant Pathology Department (Genetic Branch), Agriculture Faculty, Damanhour University, Egypt*
[*]Corresponding author's Email: mm.rahman@agr.dmu.edu.eg; ORCID: 00000003-0141-1568

Received: 10 Nov. 2020
Accepted: 21 Dec. 2020

ABSTRACT

Breeding programs play an important role in increasing the performance of chickens. The poultry industry regards growth and reproduction as the two most economically valued characteristics for providing adequate animal proteins. Genetic variations are the basis of animal breeding. The present study was conducted on three genes, including Prolactin, 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase (HMGCR), and Gonadotropin-Releasing Hormone Receptor (GNRHR). DNA was isolated from 48 chickens taken from three strains Lohmann Brown (17), Sinai (24), and Gimmizah (7) for Prolactin, HMGCR, and GNRHR gene amplification by using the PCR protocol. Electrophoresis was performed on the PCR products and the bands were viewed on a transilluminator. The size of the Prolactin gene, HMGCR, and GNRHR were 154, 675, and 210 bp, respectively. For the Sinai strain, five bands for Prolactin, two bands for HMGCR, and six bands for GNRHR were found while for the Lohmann Brown strain, five bands of Prolactin gene**,** three bands for HMGCR, and five bands for GNRHR were found. Regarding the Gimizah strain, two bands were found for Prolactin and GNRHR genes and there was only one band for the HMGCR gene. The Lohmann Brown strain respectively matured 13 and 91 earlier than Gimizah and Sinai strains with a higher egg number during the first 90 days.

Keywords: Breeding, Chickens, Egg production, GNRHR gene, HMGCR gene, Prolactin gene, PCR

INTRODUCTION

The Prolactin (PRL) gene promoter is highly polymorphic and has significant effects on egg quality traits in poultry (Liu et al., 2010). The PRL is a single-chain polypeptide hormone that belongs to the growth hormone family of genes and is mainly synthesized by lactotrope cells of the anterior pituitary gland of all vertebrates. The PRL gene is detected to be critical for the onset and kept of these reproductive behaviors in birds (Liu et al., 2010). Egg production is the most important feature in layers, which affects the economic benefits of poultry farmers (Wolc et al., 2011). Egg production is the most important trait in layer chickens as it directly influences the benefits of the poultry industry (Zhuang et al., 2019).

Molecular genetics studies the chemical mechanisms of inheritance, as an investigation of the biochemical nature of genetic material and its role in controlling phenotypic structures (Alameri et al., 2019).

3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) is a key enzyme for cholesterol homeostasis and catalyzes the rate-limiting step in cholesterol biosynthesis (Goldstein and Brown, 1990). The HMGCR gene directly regulates serum lipoprotein metabolism via a feedback mechanism (Goldstein and Brown, 1990). The HMGCR gene plays an important role during the growth and controls the transfer of primordial germ cells (Van Doren et al., 1998). The HMGCR is an important catalyzing enzyme, which catalyzes the product of 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate through four-electron oxidoreduction, which are the precursors for the generation of cholesterol in humans and are responsible for the production of ergosterol in plants, fungi, and protozoa (Istvan and Deisenhofer, 2000; Henriksen et al., 2006; Macreadie et al., 2006). Several polymorphisms have been identified in the HMGCR gene locus (Chasman et al., 2004). The HMGCR is indicated to be a logical candidate gene for

cholesterol metabolism, which is the rate-limiting enzyme in cholesterol synthesis (Xu et al., 2010). HMG-CoA reductase plays a significant role as a resident protein in the Endoplasmic Reticulum (ER) attached to the ER membrane consisting of eight transmembrane segments (Chen et al., 2012).

HMGCR gene is associated with chicken egg production (Han et al., 2014). The chicken HMGCR gene is located on chromosome Z, and also contains 20 exons and 19 introns. The HMGCR gene is important for the animal growth performance and metabolism of cholesterol. Wei et al. (2012) found that there is a relationship between the HMGCR gene and carcass traits, growth traits, and lipid profile in chickens.

Neuropeptide Y (NPY) and Gonadotrophin Releasing Hormone Receptor (GNRHR) are also two candidate genes that play an important role in physiological functions in growth, especially in reproduction processes (Fatemi et al., 2012). Two candidate genes, Gonadotropin-Releasing Hormone I (GNRH I) and Gonadotropin-Releasing Hormone II (GNRH II) play an important role in egg production in chickens (Bhattacharya et al., 2019). The GNRHR is a decapeptide released by the hypothalamus that regulates reproduction in most vertebrates. The GNRHR gene is one of the rhodopsin hormones and is expressed in the pituitary gland, the brain, and testes, spleen, and the heart (Carolsfeld et al., 2000). The GNRHR is related to the total egg production and the age of the first egg. The Gonadotropin-Releasing Hormone (GnRH) stimulates the release of gonadotropins from the pituitary gland through its receptor (Sun et al., 2001).

The production process of the avian egg is controlled by the hypothalamic-pituitary-gonadal-axis (Kuo et al., 2005). However, GNRHRs are mainly associated with the development and function of the reproductive axis in avian species (Bedecarrats et al., 2006). The GnRH binding to its receptor stimulates gonadotropin secretion from the pituitary gland and then induces the steroid production process in the gonads and egg production in chickens (Sonez et al., 2010). The GnRH and its receptor result in the appropriate growth, maturation, and maintenance of the gonads (Dunn et al., 2004). The GNRHR encoded gene is located on the long arm of chromosome number 10 and consists of 4 exons and a length of 2308 bp (NCBI).

The current experiment aimed to evaluate the reproductive traits of three chicken strains, including Lohman Brown, Sinai, and Gimmizah strains, raised in Egyptian environmental conditions. Moreover, three associated genes and related egg production traits were identified.

MATERIALS AND METHODS

Ethical approval

The present study was conducted at the Poultry Research unit (El-Bostan Farm), Department of Animal and Poultry Production, Faculty of Agriculture, Damanhour University, Damanhour, Egypt in 2018. This study was approved by the Experimental Animal Ethics Committee of the Faculty of Agriculture, Damanhour University, Egypt.

Husbandry of flocks

Three chicken strains, including Lohmann Brown (LB), Sinai (SI), and Gimmizah (GM), were raised in Egyptian environmental conditions and were selected for the reproductive traits evaluation. On the day of hatching, all chickens were permanently identified by wing banded and placed in floor incubators for the first week after hatching at a starting temperature of 33°C and then reduced by 2-3°C every week. All chickens were housed in the same room and had similar management and environmental conditions throughout the experimental period. At 18 weeks of age, the females were placed in individual laying cages (20 × 45 × 40 cm). All chickens were fed *ad libitum* on a diet containing 21% crude protein and 2.9 Kcal Metabolizable Energy (ME) /kg feed up to 6 weeks of age. They were then given a diet, containing 18% crude protein and 2.8 Kcal ME/Kg feed until they were 18 weeks old. Afterwards, they received a diet that contained 16% crude protein, 2.75 Kcal ME/Kg feed, 3.5% Ca, and 0.5% available phosphors during the egg production period. The light intensity decreased from 8-18 weeks of age to 8-10 hours, after that the light intensity increased to 16 hours per day during the laying period. The chickens were vaccinated against Newcastle Disease Virus, Gumboro, and Fowlpox diseases as recommended. At 30 weeks of age, wing vein blood samples were taken from chickens randomly from each genotype (strain).

Egg production traits

Age at Sexual Maturity (ASM) was recorded for each chicken, the period from hatching to the day of laying the first egg. The duration of laying time of the first 10 eggs and the weight (EW10) were determined as the number of days each chicken needed to give its first 10 eggs. Egg Number (EN90) and Weight (EW90) were recorded for each chicken during the first 90 days of laying. Egg Mass (EM) was measured for each chicken during the first 90 days of laying.

DNA extraction and primer design for three genes

Chicken genomic DNA was extracted from the blood samples using a Norgen Biotek kit, then quantified utilizing a spectrophotometer (pg T80, UK), and the final concentration for polymerase chain reaction (PCR) reaction was 50-100 ng/ul (Table 1). PCR primers were designed with NCBI and primers.

Table 1. Detail information for the primers of the candidate genes (GnRHR, PRL, and HMGCR) used for the polymerase chain reaction reaction.

Gene /ID	Location	Primers	Product size
GnRHR NC_006097.4 ID: 427517	Chromosome 10	F 5' CAGGGGACAGGGTGACCTA3' R 5'GAGGACCACGAGGGATGTTC3'	210 bp
PRL NC_006089.5 ID: 396453	Chromosome 2	F5'TTTAATATTGGTGGGTGAAGAGAC3' R 5'ATGCCACTGATCCTCGAAAACTC3'	154 bp
HMGCR NC_006127.4 ID: 395145	Chromosome Z	F 5'AGGGAACCTCCTGTGTTCCT3' R 5'AATGGACTTCGACTTGTGGGA3'	675 bp

GnRHR: Gonadotropin-Releasing-Hormone Receptor, PRL: Prolactin, HMGCR: 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase, bp: base pair.

Table 2. The polymerase chain reaction program used for candidate genes.

Gene	Pre - Denaturation	Denaturation	Annealing	Extension	Final Extension
GnRHR	95 ^{o}C/ 5minutes	95 ^{o}C/ 45 seconds	59.9 ^{o}C / 30 seconds	72 ^{o}C/ 45 seconds	72 ^{o}C/ 5 minutes
PRL	95 ^{o}C/ 5minutes	95 ^{o}C/ 45 seconds	54.9 ^{o}C / 30 seconds	72 ^{o}C/ 45 seconds	72 ^{o}C/ 5 minutes
HMGCR	95 ^{o}C/ 5minutes	95 ^{o}C/ 45 seconds	60 ^{o}C / 30 seconds	72 ^{o}C/ 45 seconds	72 ^{o}C/ 5 minutes

GnRHR: Gonadotropin-Releasing-Hormone Receptor, PRL: Prolactin, HMGCR: 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase.

Polymerase chain reaction amplification for the candidate genes

The PCR amplification was performed in 25 ul reaction volumes, containing master mix 2× PCR primers and DNA (Promega, Madison, USA). As can be seen in Table 2, the program for all genes was done in 35 cycles. The amplified PCR products were separated by electrophoresis on 1.5 % agarose in 1× Tris- Acetic – EDTA(TAE) buffer, ethidium bromide at the voltage of 70 volts for 1 hour, and then were read with UV light from the transilluminator (Nippon Genetics European Union).

Statistical analysis

The data of chickens from three strains of SI, LB, and GM strain were collected. The numbers of birds were 150, 130, and 101 from SI, LB, and GM pullets strain, respectively. Data analysis for egg production were performed using PROC GLM in SAS (2004) and the following formula.

$$Y_{ij} = \mu + S_j + e_{ij}$$

Where, Y_{ij} denotes observation, μ refers to general mean, Sj is the effect of strain, and e_{ij} counts for random error. The student Newman keuls option was used to test the differences between the strains. In the current experiment, ANOVA test was used to find out the significance of the experiment results. In other words, the test helps to figure out whether the null hypothesis needs to be rejected or the alternate hypothesis can be accepted under probability ($p \leq 0.01$).

RESULTS

The egg production

The least-square means for egg production traits in the three strains of Lohmann Brown (LB), Gimmizah (GM), and Sinai (SI) are indicated in Table 3. The LB strain matured significantly younger ($p \leq 0.01$) (in 147.5 days) than those of local GM and SI strains (Table 3). In addition, Sinai pullets matured earlier (in 156.6 days), compared to the Gimmizah strain (in 160.8 days).

Lohmann Brown Pullets matured 13.3 days and 9.1 days earlier than the GM and SI pullets, respectively. Regarding ASM, there was a difference between the three strains, possibly due to genetic makeup. In a breeding program, ASM is a very important trait as it affects the properties of egg production.

The duration of laying the first 10 eggs (DU 10) is presented in Table 3. There was a highly significant difference ($p \leq 0.01$) between the strains ranging from 13.5 to 17.3 days. The required time to lay the first 10 eggs of LB pullets is shorter than SI and GM pullets. The SI strain indicated a decreased in DU 10, compared to the GM strain by 2.18 days. The average egg weight of the first 10 eggs in the LB strain was 51.12 g, compared to 48.5 gm in the GM strain. The SI strain indicated a decrease in the average egg weight of the first 10 eggs of 42.7 g. The egg counts during the first 90 days were 62.45, 45.07, and 43.27 for the LB, GM, and SI strains, respectively (Table 3). The Lohmann Brown strain had a highly significant EN90 than the GM and SI strains. On the other hand, there was no significant difference in EN90 between GM and SI strain.

The Total mean of LB strain indicated a highly significant ($p \leq 0.01$) increase in average egg weight during the first 90 days (57.58g), compared to GM strain (53.81 g) and SI strain (48.74 g). The Gimmizah pullets had the highest egg weight, compared to the SI pullets in the first 90 days and the difference was significant ($p \leq 0.01$). Table 3 presents a highly significant difference ($p \leq 0.01$) between strains on egg mass during the first 90 days of laying. Pullets of LB strain produced significantly the largest egg mass, compared to the other pullet strain. The overall means of egg mass during the first 90 days were 3633.69, 2459.95, and 2099.19 g for LB, GM, and SI strain, respectively. The SI strain indicated a lower egg mass than the other strains.

Table 3. Least square means ± Standard Error and analysis of variance for egg production traits in three strains of Lohman Brown, Gimmizah, and Sinai

Trait \ Strain	GM	` SI	LB	ANOVA
Age & sexual maturity	160.89 ± 2.22^{A}	156.66 ± 1.01^{B}	147.50 ± 0.69^{C}	**
Duration of layingthe first10 eggs	17.30 ± 0.73^{A}	15.12 ± 0.21^{B}	13.59 ± 0.25^{C}	**
Egg weight at first 10 eggs	48.50 ± 0.59^{B}	42.74 ± 0.42^{C}	51.12 ± 0.32^{A}	**
Egg number during first 90 days of laying	45.07 ± 2.53^{B}	43.27 ± 0.84^{B}	62.45 ± 1.18^{A}	**
Egg weight at first 90 days of laying	53.81 ± 0.81^{B}	48.74 ± 0.79^{C}	57.58 ± 0.53^{A}	**
Egg mass	2459.95 ± 156.80^{B}	2099.19 ± 47.88^{C}	3633.69 ± 87.09^{A}	**

Different letters in each row mean significant differences ($p \leq 0.05$). **: significantly different between strains at ($p \leq 0.001$). GM: Gimmizah, SI: Sinai, LB: Lohman Brownç

Polymerase chain reaction amplification for Prolactin gene

The Prolactin gene located on Chromosome 2 is the candidate gene associated with increased egg production in chickens. The Polymerase Chain Reaction (PCR) was used to amplify the Prolactin gene fragments. The amplified product was 154 bp, indicating that the amplicon was Prolactin. For the SI strain, 5 bands appeared on lines 8, 22, 26, 64, and 70 (Figure 1). For the LB strain, five bands appeared on lines 9, 12, 14, 58, and 97 (Figure 2), and also for GM two bands appeared on lines 71 and 81 (Figure 2).

Polymerase chain reaction amplification for 3-Hydroxy-3-methylglutaryl-coenzyme A reductase gene

As an important candidate gene affecting cholesterol metabolism, polymorphisms of the HMGCR gene and their associations have attracted much attention in mammals. The amplified product was 675 bp. For the SI strain, two bands were appeared on lines 8 and 22 (Figure 3), while, three bands appeared on lines 12, 14, and 97 for the LB strain (Figure 4), finally for GM strain only one band appeared on line 81 (Figure 4).

Polymerase chain reaction amplification for Gonadotropin-Releasing Hormone Receptor gene

Fragments were amplified using PCR. The amplified product was 210 bp, indicating that the amplicon was GNRHR. For SI strain, 5 bands were displayed on lines 8, 22, 26, 64, and 71 (Figure 5). For the LB strain, 3 bands were appeared on lines 12, 14, and 97 (Figure 6). For the GM strain, 2 bands were appeared on lines 71 and 81 (Figure 6). The number of genes associated with economic characters was limited and few.

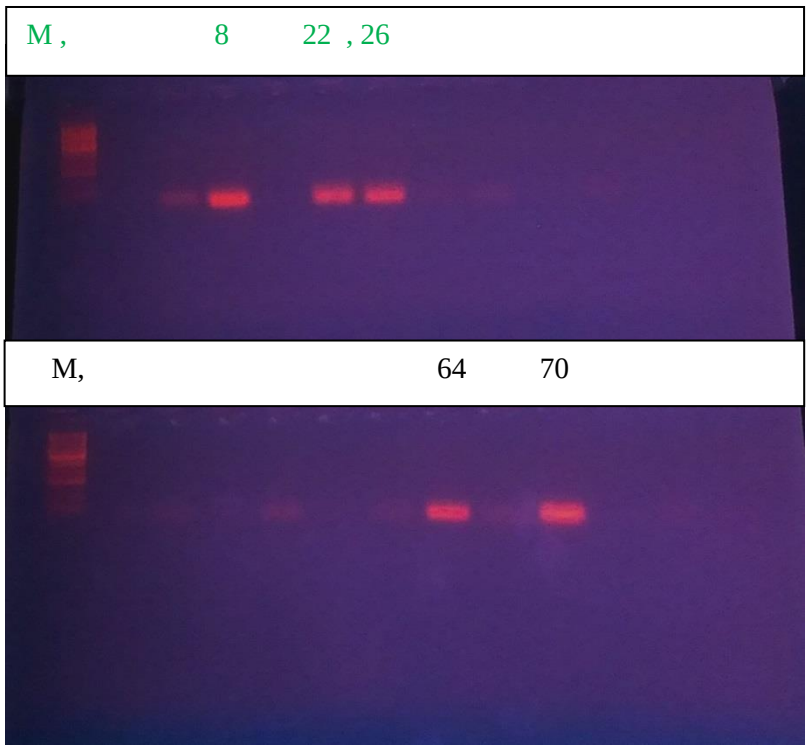


Figure 1. Polymerase chain reaction amplification gel image of the Prolactin gene of chicken (Sinai strain, M=1 kb ladder)

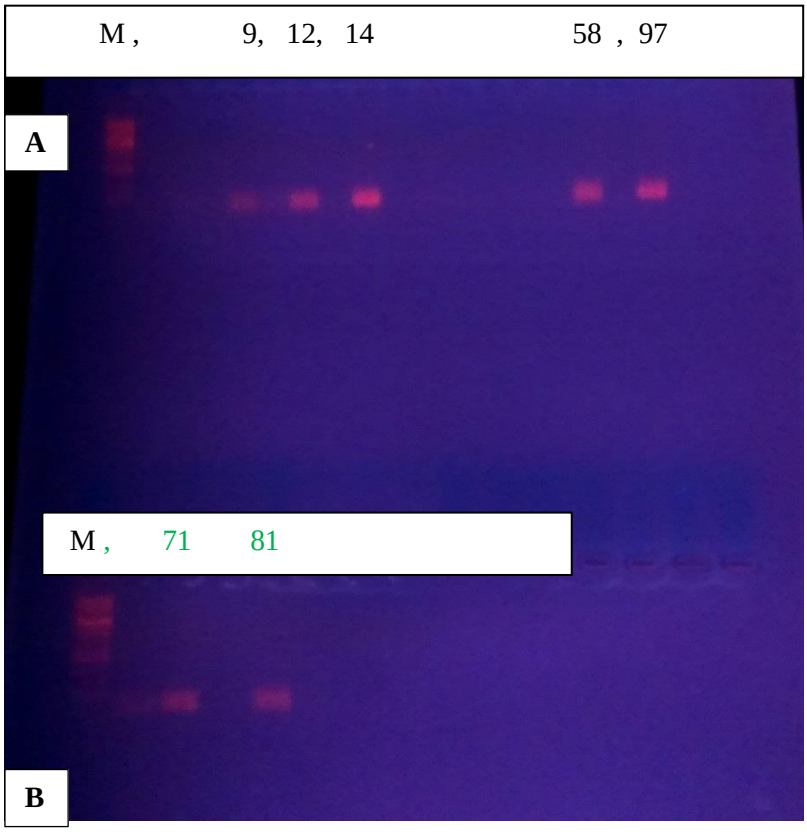


Figure 2. Polymerase chain reaction amplification gel image of the Prolactin gene of chicken (A: Lohmann Brown strain, B: Gimmizah strain, M= 100bp1kb ladder)

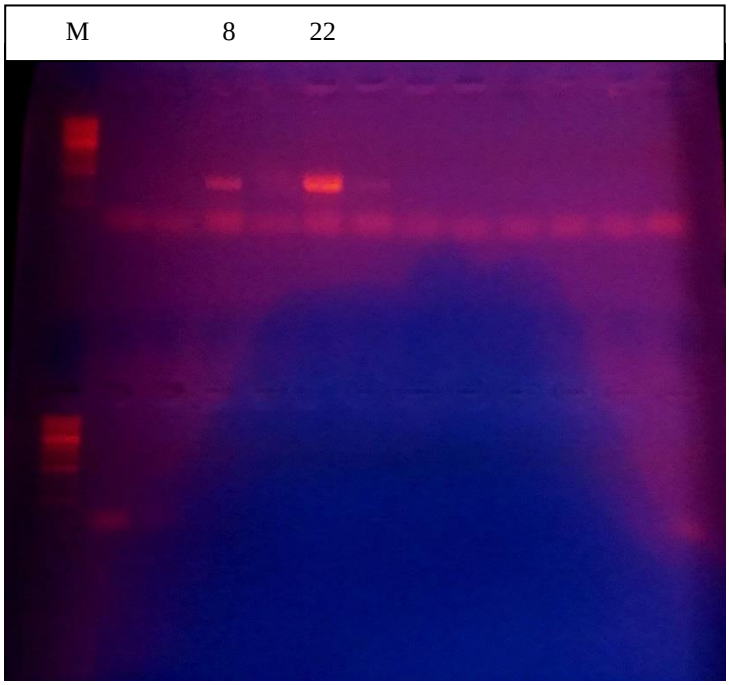


Figure 3. Polymerase chain reaction amplification gel image of the HMGCR gene of chicken (Sinai strain, M=1kb ladder)

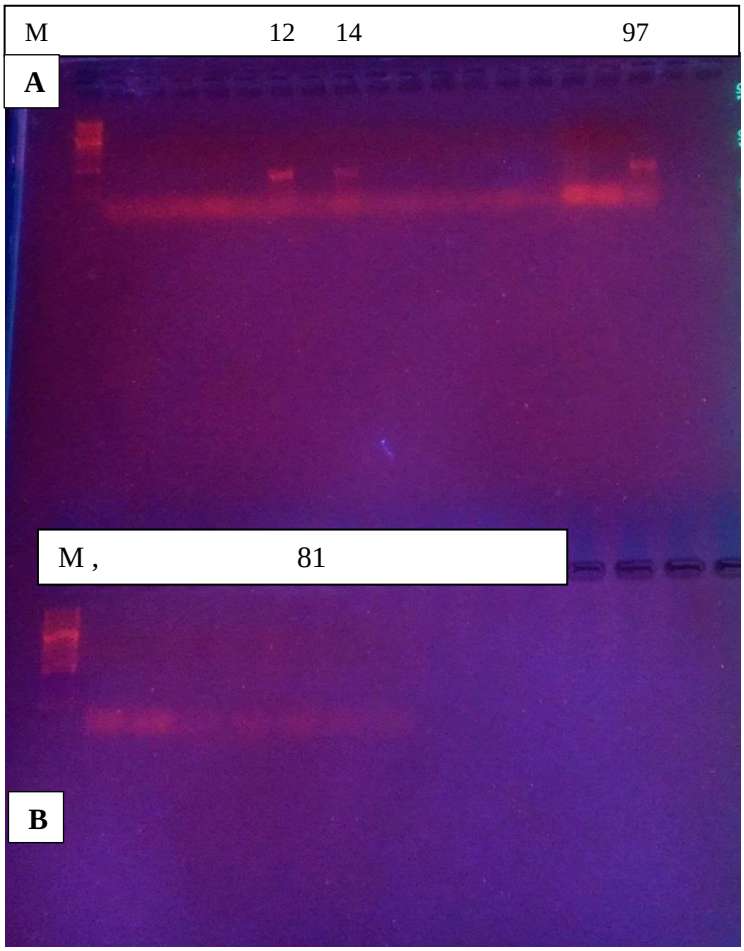


Figure 4. Polymerase chain reaction amplification gel image of the HMGCR gene of chicken. (A: Lohmann strain, B: Gimmizah strain, M= 1kb ladder)

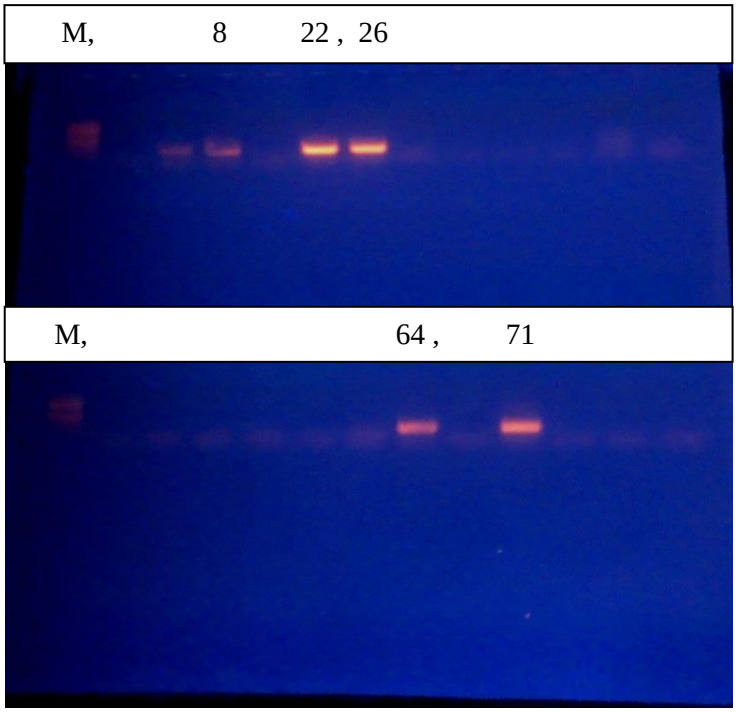


Figure 5. Polymerase chain reaction amplification gel image of the GNRHR gene of chicken (Sinai strain, M=100 bp)

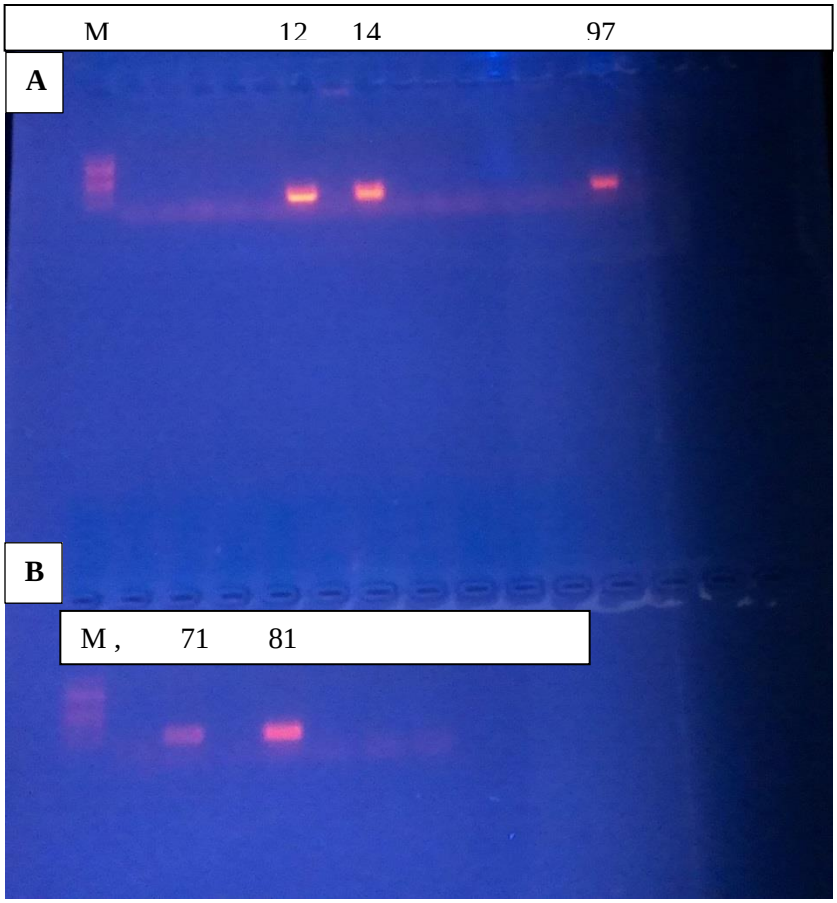


Figure 6. Polymerase chain reaction amplification gel image of the GNRHR gene of chicken (A: Lohmann Brown strain, B: Gimmizah strain, M = 100bp)

DISCUSSION

In a breeding program, ASM is a really important trait as it affects the properties of egg production. The laying duration of the first 10 eggs (DU 10) is presented in Table 3. The ASM of different strains was within the range of 13.5-17.3 days, which supported the results of studies conducted by EL-Labban et al. (2011), Ghanem et al. (2012), and EL-Tahawy (2015).

The duration of laying in the first 10 eggs (DU 10) is presented in Table 3. There was a highly significant difference ($p \leq 0.01$) between strains ranging from 13.5 to 17.3 days, which is in line with the previous studies reported by Bonekamp et al. (2010), and Taha et al. (2012). The number of eggs during the first 90 days was calculated as 62, 45, and 43 for LB, GM, and SI strains, respectively. The obtained results of maturity agreed with studies performed by Alicja Sobczak and Krzysztof, (2015), and EL-Tahawy (2015) that found the overall means of ASM 147.5 and 156.6 days for LB and SI strains, respectively. Present results in Table 3 were in agreement with those obtained by Bonekamp et al. (2010), and Alicja sobczak and Krzysztof (2015). The overall means of egg mass during the first 90 days were 3633.69, 2459.95, and 2099.19 g for LB, GM, and SI strains, respectively, which was consistent with the results reported by Soares et al. (2011), Alicja sobczak and Krzysztof (2015), and EL-Tahawy (2015).

Various studies have indicated that polymorphisms in the chicken PRL gene of different breeds are associated with egg production (Bhattacharya et al., 2011; Cui et al., 2006). Prolactin regulates important physiological functions, such as reproductive and homeostatic effects. Elkins et al. (2000), and Zhang et al. (2012) found that the polymorphism of the PRL gene was significantly associated with egg production traits in chickens and that it is a strong candidate gene affecting egg production traits.

The HMGCR was considered to be a logical candidate gene for cholesterol metabolism, as HMGCR is the rate-limiting enzyme in cholesterol synthesis (XU et al., 2010). The GNRHs are responsible for the development and function of the reproductive properties in avian (Bedecarrats et al., 2006).

CONCLUSION

In a breeding program, age at sexual maturity (ASM) is a very important trait as it affects the properties of egg production. Based on the obtained results of the current study, ASM was significantly different among the three strains of Lohmann Brown, Sinai, and Gimmizah possibly due to the genetic makeup. The Lohmann Brown strain can be used to mature earlier and have a higher egg number than Gimizah and Sinai strains during the first 90 days. The performed PCR indicated some bands for Prolactin, 3-Hydroxy-3-methylglutaryl-coenzyme A reductase, and Gonadotrophin Releasing Hormone Receptor genes. The authors of the present study aim to complete their works by examining gene expression using RT-PCR.

DECLARATION

Authors' contribution

El-Tahawy collected the samples and designed research. Abdel-Rahman was responsible for DNA extraction, PCR, and participated in writing. All authors checked and confirmed the final draft of manuscript.

Acknowledgments

Research supported by the Science and Technology Development Fund (STDF, 2418).

Competing interests

The authors of present study did not have any conflict of interests.

REFERENCES

Alameri MS, Al-anbari EH, and Razuki WM (2019). Association the gonadotropin-releasing hormone receptor (GNRHR) gene polymorphisms with egg production traits in Iraqi local brown chicken.Biochemical. Cellular. Archives, 19(1): 1373-1380. DOI: https://www.doi.org/10.35124/bca.2019.19.1.1373

Alicja Sobczak A, and Krzysztof K (2015). The effect of a probiotic preparation containing bacillus subtilisatccpta- 6737 on egg production and physiological parameters of laying hens. Annual Animal science, 15(3): 711-723. DOI: https://www.doi.org/10.1515/aoas-2015-0040

Bedecarrats GY, Shimizu M, and Guemene D (2006). Gonadotropin-releasing hormones and their receptors in avian species. PoultryScience, 43: 199-214. DOI: https://www.doi.org/10.2141/jpsa.43.199

Bhattacharya TK, Chatterjee RN, Dange M, and Bhanja SK (2019). Polymorphisms in GnRHI and GnRHII genes and their association with egg production and egg quality traits in chicken.

British Poultry Science, 60: 187-194. DOI: https://www.doi.org/10.1080/00071668.2019.1575505

Bhattacharya TK, Chatterjee RN, Sharma RP, Rajkumar U, Niranjan M, and Reddy BLN (2011). Association of polymorphism in the prolactin promoter and egg quality traits in laying hens. British Poultry Science, 52(5): 551-557. DOI: https://www.doi.org/10.1080/00071668.2011.617727

Bonekamp RP, Lemme RTA, Wijttend PJA, and Sparla JKW (2010). Effect of amino acids on egg number and egg mass of brown (heavy breed) and white (light breed)laying hens. Poultry Science, 89: 522-529. DOI: https://www.doi.org/10.3382/ps.2009-00342

Carolsfeld J, Powell J, Park M, Fischer W, Craig A, Chang J, Rivier J, and Sherwood N (2000). Primary structure and function of three gonadotropin releasing hormones, including a novel form, from an ancient teleost, herring. Endocrinology, 141: 505-512. DOI: https://www.doi.org/10.1210/endo.141.2.7300

Chasman D, Posada D, Subrahmanyan L, Cook N, Stanton V, and Ridker P (2004). Pharmacogenetic study of statin therapy and cholesterol reduction. The Journal of the American Medical Association, 291: 2821-2827. DOI: https://www.doi.org/10.1001/jama.291.23.2821

Chen X ,Wang X, Li Z, Kong L, Liu G, Fu J, and Wang A (2012). Molecular cloning tissue expression and protein structure prediction of the porcine 3-hydroxy-3-methylglutaryl coenzyme a reductase (HMGR) gene Gene, 495: 170-177. DOI: https://www.doi.org/10.1016/j.gene.2011.12.051

Cui J, Du H, Liang Y, Deng X, Li N, and Zhang X (2006). Association of polymorphisms in the promoter region of chicken prolactin with egg production. Poultry Science, 85(1): 26-31. DOI: https://www.doi.org/10.1093/ps/85.1.26

Dunn IC, Miao YW, Morris A, and Romanov MN (2004). A study of the association between genetic markers in candidate genes and reproductive traits in one generation of a commercial broiler breeder hen population. Heredity, 92: 952-957. DOI: https://www.doi.org/10.1038/sj.hdy.6800396

Elkins PA, Christinger HW, Sandowski Y, Sakal E, Gertler A, de Vos AM, and Kossiakoff AA (2000). The ternary complex between placental lactogen and the extracellular domain of the prolactin receptor. Nature . Structural. Biology, 7: 808-815. DOI: https://www.doi.org/10.1038/79047

EL-Labban AM, Iraqi MM, Hanafi MS, and Heba AH (2011). Estimation of the genetic and non-genetic parameter for egg production trait in a local strain of chicken. Livestock Research for rural development 23: Article ID #10. Available at: http://www.lrrd.org/lrrd23/1/ella23010.htm

EL-Tahawy WS (2015). Effect of strain on some growth and egg production traits between two strains of chicken under Egyptian environmental conditions. Egyptian Poultry Science, 35: 1137-1145.

Fatemi SA, Mehrabani- Yeganeh H, Nejati- Javaremi A, and Niknafs SH (2012). Association of neuropeptide Y and gonadotrophin-releasing hormone receptor gene SNPs with breeding value for growth and egg production traits in Mazandaran native chickens Genetics and Molecular Research, 11(3): 2539-2547. DOI: https://www.doi.org/10.4238/2012.july.10.9

Ghanem HH, EL-Tahawy WS, Attia YA, and Nawar AN (2012). Devolping A 3 - way cross of chickens to improve egg production traits. Egyptian Poultry Science, 32: 547-560. Available at: https://scholar.google.com/scholar?cluster=9414398906143841801&hl=en&as_sdt=2005&sciodt=0,5

Goldstein J, and Brown M (1990). Regulation of the mevalonate pathway. Nature, 343: 425-430. DOI: https://www.doi.org/10.1038/343425a0

Han C, An G, and Du X (2014). Three novel single nucleotide polymorphisms of the 3-hydroxy-3-methylglutaryl coenzyme a reductase gene associated with egg-production in chicken. Folia Biologica (Kraków), 62: 203-209. DOI: https://www.doi.org/10.3409/fb62_3.203

Henriksen J, Rowat AC, Brief E, Hsueh YW, Thewalt JL, Zuckermann MJ, and Ipsen JH (2006). Universal behavior of membranes with sterols. Biophysical. Journal, 90: 1639-1649. DOI: https://www.doi.org/10.1529/biophysj.105.067652

Istvan E, and Deisenhofer J (2000). The structure of the catalytic portion of human HMG-CoA reductase Biochimica et Biophysica Acta, 1529(1-3): 9-18. DOI: https://www.doi.org/10.1016/s1388-1981(00)00134-7

Kuo Y, Shiue YL, Chen CF, Tang PC, and Lee YP (2005). Proteomic analysis of hypothalamic proteins of high and low egg production strains of chickens Theriogenology, 64: 1490-1502. DOI: https://www.doi.org/10.1016/j.theriogenology.2005.03.020

Liu WJ, Sun DX, Yu Y, Li G, Tang SQ, Zhang Y, and Wang YC (2010). Association of Janus kinase 2 polymorphisms with growth and reproduction traits in chickens. Poultry Science, 89(12): 2573-2579. DOI: https://www.doi.org/10.3382/ps.2010-00988

Macreadie I, Johnson G, Schlosser T, and Macreadie P (2006). Growth inhibition of Candida species and Aspergillus fumigatus by statins FEMS Microbiology Letters, 261(1): 9-13. DOI: https://www.doi.org/10.1111/j.1574-6968.2006.00370.x

SAS (2004). USM, S Guide: Statistics SAS, Institute inc. Cary, NS, U.S.A.

Sun YM, Dunn IC, Baines E, Talbot RT, Illing N, Millar RP, and Sharp PJ (2001). Distribution and regulation by oestrogen of fully processed and variant transcripts of gonadotropin releasing hormone I and gonadotropin releasing hormone receptor mRNAs in the male chicken. Journal of Neuroendocrinol, 13: 37-49. DOI: https://www.doi.org/10.1111/j.1365-2826.2001.00587.x

Sonez M, Sonez C, Mugnnaini M, and Haedo M (2010). Effects of differential pulse frequencies of chicken gonadotrophin- releasing hormone -1(cGnRH-1) on laying hen gonadotrope responses *in vitro*. Biotechnology Histochemistry, 85: 355-363. DOI: https://www.doi.org/10.3109/10520290903368774

Soares ML, Lopes JC, Brito NV, and Carualherira J (2011). Characterization of growth and egg production of two Portuguese autochthonous chicken breed pretalusitanica and amarela. The 62nd annual meeting of the European Association for Animal production-August 29th to September 2nd, Stavanger, Norway. DOI: https://www.doi.org/10.4081/ijas.2015.3566

Taha AE, Abdel ghany FA, and Sharaf MM (2012). Strain effect on some productive and reproductive performance traits of local improved Egyptian and Canadian chickens. Journal of animal and feed research, 2: 292-300. Available at: https://www.ojafr.ir/main/attachments/article/87/OJAFR,%20B55,%20292-300,%202012.pdf

Xu N, Taylork D, Azziz R, and Goodarzi MO (2010). Variants in the HMG-CoA reductase (HMGCR) gene influence component phenotypes in polycystic ovary syndrome. Fertility. Sterility, 94: 255-260. DOI: https://www.doi.org/10.1016/j.fertnstert.2009.01.158

Van Doren M, Broihier H, Moore L, and Lehmann R (1998). HMG-CoA reductase guides migrating primordial germ cells. Nature, 396: 466-469. DOI: https://www.doi.org/10.1038/24871

Wei Y, Zhu S, Zhang S, Han R, Tian Y, Sun G, and Kang X (2012). Two novel SNPs of the 3-hydroxy-3-methylglutaryl coenzyme a reductase (HMGR) gene associated with growth and meat quality traits in the chicken. Genetics and Molecular Research, 11: 4765-4774. DOI: https://www.doi.org/10.4238/2012.November.12.10

Wolc A, Arango J, Settar P, O'Sullivan NP, and Dekkers JC (2011). Evaluation of egg production in layers using random regression models. Poultry Science, 90(1): 30-34. DOI: https://www.doi.org/10.1016/j.fertnstert.2009.01.158

Zhang L, Li D, Liu Y, Wang Y, Zhao X, and Zhu Q (2012). Genetic effect of the prolactin receptor gene on egg production traits in chickens Genetics and molecular research, 11: 4307-4315. DOI: https://www.doi.org/10.4238/2012.October.2.1

Zhuang L, Ning Y, Yiyuan Y, Guangqi L, Aiqiao L, Guiqin W, and Congjiao S (2019). Genome-wide association analysis of egg production performance in chickens across the whole laying period BMC, Pp. 1-9. DOI: https://www.doi.org/10.1186/s12863-019-0771-7.

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 615-622, December 25, 2020

Research Paper, PII: S2322455X2000070-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.70

The Effect of Dietary Administration of Virgin Coconut oil on Differential Leukocytes in Infected Chicken with *Eimeria tenella*

Zakia Sheila Faradilla[1], Muchammad Yunus[1*], and Herry A. Hermadi[2]

[1]*Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia*
[2]*Department of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia*

*Corresponding author's Email: muhyunus_99@yahoo.com, ORCID: 0000-0001-7516-6628

Received: 03 Nov. 2020
Accepted: 18 Dec. 2020

ABSTRACT

Coccidiosis is the main problem in poultry diseases. It is caused by the parasite *Eimeria tenella,* which induce the immune response of leukocyte. Anticoccidial drugs are administered in the poultry feed to control coccidiosis. However, taking medication for a long time can lead to resistance. Recent studies have indicated that Virgin Coconut Oil (VCO) has some benefits, including anti-inflammatory effects. The present research aimed to identify the effect of VCO at the different doses in improving the various leukocyte counts of chickens infected with *E. tenella*. Male chickens were divided into five groups (T0, T1, T2, T3, and T4) and treated for 28 days. T0 was neither infected with *E. tenella* nor get treatment, T1 was only infected with *E. tenella*, T2 was treated with 5 ml/kg VCO feed and had *E. tenella* infection, T3 was treated with 10 ml/kg VCO feed and had *E. tenella* infection, and T4 was treated 20ml/kg VCO feed and had *E. tenella* infection. Differential leukocyte was counted with a blood cell counter. The data obtained were analyzed using ANOVA and Duncan's Multiple Range Test. The results indicated that a dose of 10 ml/kg feed and 20ml/kg feed of VCO could improve the differential leukocyte counts of chickens infected with *E. tenella* by maintaining the counts of basophil, eosinophil, heterophil, monocyte, and lymphocyte in the normal range. The present study concluded that VCO by a dose of more than 10 ml/kg would improve the differential leukocyte counts of chickens infected with *E. tenella*.

Keywords: Differential leukocyte count, *Eimeria tenella*, Virgin Coconut Oil

INTRODUCTION

Coccidiosis caused by the parasite *Eimeria tenella* is the main problem facing poultry farmers. This parasite has a high virulence and multiplies intracellularly in the digestive tract of chicken caecum (Habibi et al., 2016; Fatoba and Adeleke, 2018). Coccidiosis is a disease caused by protozoa in a phylum of Aplicomplexa and genus *Eimeria*. Coccidiosis causes damage to the gastrointestinal tract and primarily affects domestic animals, wild animals, and poultry (Debbou-Iouknane et al., 2018; Dubey, 2018; Bachene et al., 2019). Although coccidiosis is a disease that was recognized long ago, the impact of economic loss because of it causing coccidiosis is becoming an important issue in society (Chapman, 2008; Debbou-Iouknane et al., 2018).

The economic loss caused each year by the coccidiosis in a poultry industry around the world reaches about US$ one billion, due to the decrease in poultry performance and an increase in production cost (Berezin et al., 2010). Seven species of *Eimeria* (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. nectrix, E. praecox,* and *E. tenella*) were known to be pathogenic in chickens (Chengat Prakashbabu et al., 2017), Among all *Eimeria* species, however, *E. tenella* is the most pathogenic species in which the predilection lies in the caecum and causes caecal coccidiosis (Muazu et al., 2008). Coccidiosis is controlled by adding anticoccidia to the feed (Pop et al., 2019). Recently, the usage of anticoccidia is still the main choice in the poultry industry as drugs administration is more effective than just depending on the poultry immune response (Alfaro et al., 2007; Pop et al., 2019). However, regular using of anticoccidial drugs over a long period of time had caused coccidian to become resistance to the drug, so the use of anti-coccidia and antibiotics must be reduced (Tipu et al., 2002).

There is a specific system in the body to eradicate various infectious and toxic materials. So, the immune system consisted of leukocyte (white blood cells) and tissue cells that derive from leukocyte. A disruption of the immune system leads to a change in the immune function,

especially the cellular immune system such as leukocyte (Roeslan, 1996). Therefore, the leukocyte count of calculations can be used as one of the immune response indicators to determine if there is an infection in the body (Abdul Mohymen et al., 2014).

Virgin Coconut Oil (VCO) is a dietary supplement which can be produced by Indonesian coconut farmers in the home industry and is proved secure to be consumed by a human. Hence, it is believed that VCO can also be useful as a feed additive to be consumed by chickens (Attia et al., 2020). Recent studies have shown those VCO exhibits varying degrees of beneficial properties, such as antiviral, antibacterial, antifungal, anti-inflammatory, antipyretic, and antioxidant properties (Akinnuga et al., 2014). Virgin Coconut Oil (VCO) has proven to have a broad spectrum of antimicrobial activities against pathogenic microorganisms, including Gram-positive bacteria, i.e. *S.aureus*, *Listeria monocytogenes*, *Streptococcus pyogenes*, and *Bacillus cereus*; Gram-negative bacteria, i.e., *Escherichia coli*, *Vibrio cholera* and *Salmonella* Typhi and yeast, i.e. *Candida albicans*, *C.krusei* and *Pityrosporumovale* (Long, 1968).

In addition, a recent study on the effect of VCO against coccidiosis in broiler chicken indicated promising results and could be used as an alternative method to control coccidiosis in the poultry industry (Tan and Long, 2012). So, the objective of the present study was to evaluate the effect of VCO in improving the differential leukocyte count of broiler chicken infected with *E. tenella*.

MATERIALS AND METHODS

The present research used some equipment. There was chicken cage, feed and drink media, label, scale, media for gems, Slide, microscope, syringe, beaker glass, spuit, petri dish, cover dish, centrifuge, pipet, cover glass, and Blood Cell Counter.

The present research used *Eimeria tenella* from the Parasitology Department of Universitas Airlangga, DOC (Day Old Chick) strain Cobb from the chicken supplier in Surabaya, 14 days old chicken with Specific Antibody Negative (SAN), potassium dichromate (K2Cr2O7) 2.5%, sugar solution 96%, equates, Giemsa 10%, distilled water, methanol and Virgin Coconut Oil (VCO) from Vico Bagoes by dr. Zainal Gani Jakarta.

Ethical Approval

Ethical regulations in the present study from the perspective of an animal breeder and researcher (chicken cage, feed drink media and handlings) were in according to "Bioethics of Poultry Production" (Macer, 2019).

Activation and multiplication of *Eimeria tenella* oocysts

Eimeria tenella isolate was activated by infecting five chickens at the age of two weeks that had never been infected by coccidiosis with 10,000 doses each. The chicken feces were collected on the seventh day after infection until the chickens were slaughtered on day tenth day. The feces were then mixed and placed in 2.5% potassium dichromate (K2Cr2O7) medium at room temperature (28° C) for 24-48 hours for the sporulation process, so that the infectous oocysts of *E. tenella* were obtained.

The sugar solution was mixed with the feces that has been speculated in the 2.5% potassium dichromate and placed in a beaker glass at the ratio of 1/1. The solution was stirred until completely homogenous and then poured into a petri dish. After five minutes and the oocysts rose to the surface, the petri dish closed with the cover dish. After 30 minutes cover dish was lifted and washed with equivalents including distilled water. The oocyst suspension was then poured into a beaker glass. The isolation was done 5 to 6 times. The oocyst suspension, which still contained sugar, was washed with distilled water and centrifuged at 2500 rpm for 5 to 10 minutes. This process was performed 3-4 times until the oocyst suspension became clear.

The oocysts count was performed using Haemositometer-Improved-Neubauer. The oocysts were observed and calculated under a microscope with 100× magnification. The calculation was made from the oocysts on four large squares in the corner of the courtroom. The number of oocysts from each milliliter suspension was therefore 2.5 × 1000 n = 2500 n. The desired infection dose was prepared by diluting, so that each milliliter contained the required number of oocysts.

Treatment of chickens

The experimental unit that was used in the present study was healthy male Day-Old Chickens (DOCs) from a chicken supplier in Surabaya. The chickens' diets were standard feed for starter (0 to 7 days) and grower (8 to 34 days) diets, without coccidiostats additive (Tan and Long, 2012).

Samples of 30 DOCs were divided into five groups, the controlled negative (T0) group, the control positive (T1) group, the treatment I (T2) group, the treatment II (T3) group, and the treatment III (T4) group. In the control negative (T0) group, the chicken was not infected

with *E. tenella* and was not treated with VCO. The chicken's cage was separated from the control positive (T1) group and treatment (T2, T3, and T4). In the control positive (T1) group the chicken was only infected with *E. tenella* at the age of 21 days old but was not treated with VCO. In the group of treatment I (T2), the chicken was treated with five ml VCO/kg feed from one day old chicken up to 28 days and infected with *E. tenella* at the age of 21 days old. In the group of treatment II (T3), the chicken was treated with 10 ml VCO/kg feed from one-day old chick up to 28 days and infected with *E. tenella* at the age of 21 days old. In the group of treatment III (T4), the group was treated with 20 ml VCO/kg feed from one-day old chick up to 28 days and infected with *E. tenella* at the age of 21 days.

Blood sampling and smear preparation

Blood sampling was done on hatch day, day three, and day six after infection. The blood was taken from the brachialis vein and then the blood smear was prepared. One drop of the chicken blood sample was anticoagulant in the first slide with a flat position. Then, the slide with blood sample was shifted using another sterile slide with an angel of 30° so that it formed a thin layer of blood and it was dried at environment temperature. The blood smear was then soaked into methanol as a fixation within five minutes. The blood smear was dried at the environment temperature and then soaks into gems stain for 30 minutes. Thereafter, the blood smear was washed with water after staining, then the blood smear was placed vertically and allowed to dry again. Then a drop of Immersion oil was dropped on the blood smear and was observed under a microscope with 1000× magnification. The count started in the count area from the top left to bottom, then shifted to the right and then to the up and repeated until 100 cells of leukocyte were gotten based on the types using blood cell counter.

Data analysis

The design of the present research used completely randomized design. The data were analyzed by analysis of variance (ANOVA) and then followed by Duncan's Multiple Range Test to compare the treatment effect of each treatment.

RESULTS

The leukocytes count in each treatment group was performed from the blood smear. These five distinct leukocytes were basophil, eosinophil, heterophil, monocyte, and lymphocyte. The data on the leukocyte count were given as follows. The results of basophil count from the blood smear from *E. tenella* infected chicken that had been treated with VCO are presented in table 1.

The data analysis of the VCO addition in the feed indicated that there was no significant difference ($p < 0.05$) in the basophil count. The Duncan's Multiple Range Test indicated that the infected group had the highest decreasing amount of basophil among other groups on the day 6th after infection. The 3rd day after the infection group had the highest amount of basophil count and the highest number that could have got was from the T4 group with one basophil cell. The sixth day after the infection, infected group had less amount of the basophil and the lowest number that could have got was from the T1, T3, and T4 group with no basophil cell found. The results of eosinophils count from the blood smear of chicken treated with the VCO are presented in table 2.

Table 1. The result of the basophil count of the VCO effect in chicken infected with *E. tenella*

Treatment	Day after infection		
	0	3	6
T0	$0.50^{a} \pm 1.00$	$0.50^{a} \pm 0.58$	$0.25^{a} \pm 0.50$
T1	$0.75^{a} \pm 0.96$	$0.75^{a} \pm 0.50$	$0.00^{a} \pm 0.00$
T2	$0.25^{a} \pm 0.50$	$0.50^{a} \pm 0.58$	$0.25^{a} \pm 0.50$
T3	$0.25^{a} \pm 0.50$	$0.25^{a} \pm 0.50$	$0.00^{a} \pm 0.00$
T4	$0.50^{a} \pm 0.58$	$1.00^{a} \pm 1.15$	$0.00^{a} \pm 0.00$

[a:] Means within a column with different superscripts differ significantly ($p < 0.05$). T0: negative control, T1: positive control or infected with *E. tenella*, T2: 5 ml/kg feed of VCO, T3: 10 ml/kg feed of VCO, T4: 20 ml/kg feed of VCO.

Table 2. The result of eosinophil count of the VCO effect in chicken infected with *E. tenella*

Treatment	Day after infection		
	0	3	6
T0	$2.00^{b} \pm 1.41$	$3.50^{a} \pm 2.08$	$4.25^{b} \pm 1.26$
T1	$4.25^{a} \pm 1.50$	$4.25^{a} \pm 1.25$	$6.75^{a} \pm 2.50$
T2	$4.50^{a} \pm 1.73$	$4.50^{a} \pm 1.00$	$2.50^{bc} \pm 1.29$
T3	$3.25^{ab} \pm 0.50$	$4.75^{a} \pm 2.63$	$1.00^{c} \pm 1.15$
T4	$3.50^{ab} \pm 1.29$	$4.75^{a} \pm 1.26$	$1.00^{c} \pm 0.82$

[a, b, c:] Means within a column with different superscripts differ significantly ($p < 0.05$). T0: negative control, T1: positive control or infected with *E. tenella*, T2: 5 ml/kg feed of the VCO, T3: 10 ml/kg feed of the VCO, T4: 20 ml/kg feed of the VCO.

The data in table 2 indicated that there was a significantly different ($p < 0.05$) among each group which increased in the third day and decreased in the sixth day after the infection. The third day after the infection, the infected groups which had the highest number of eosinophil were T3 and T4. Otherwise, the sixth day after the infection, the groups which had the less number of eosinophil were also T3 and T4. Although the T3 and T4

were not significantly different from each other, they significantly different from other groups. T1 group was significantly different from the T2 group on the sixth day after infection. T1 group had the highest number of eosinophil among all groups on the day six after infection. Otherwise, T3 and T4 group had the lowest number of eosinophil among all other groups on the day six after infection. The results of heterophils count from the blood smear of chicken treated VCO are presented in table 3.

The data of table 3 presented that the T3 and T4 groups were significantly different ($p < 0.05$) from all other groups, although T3 and T4 group were not significantly difference with each other. The highest number of heterophil on the third day after the infection was seen in the T2 group. The lowest number of heterophil on the sixth day after the infection was seen in the T4 group. The results of Monocytes count from the blood smear of chicken treated with the VCO are presented in table 4.

The data had a significant difference ($p < 0.05$) in the T4 group. The T1 group had the highest number of monocyte on the third day after infection, while the T4 group had the lowest number of monocyte on the third day after infection. The T4 group had the lowest number of monocyte on the sixth day after infection. The results of the lymphocyte count from the blood smear of chicken was treated with the VCO are presented in table 5.

The data of table 5 indicated significant difference ($p < 0.05$) on the sixth day after the infection. The T4 group had the highest number of lymphocyte on the sixth day after infection, while the T3 group had the lowest number. The T2 and T3 groups showed significant difference at the beginning of the trial, the third, and the sixth day after infection. Although the T4 group showed a significant difference at the beginning of the trial and on the third day after infection, did not show any significant difference on the third and sixth days after infection. T1 group or control positive group showed a constantly decreasing number of lymphocytes, in contrast to other groups which showed an increasing number of lymphocytes.

Table 3. The result of the heterophil number of VCO effect in infected chicken with *E. tenella* infected chicken

Treatment	Day after infection		
	0	3	6
T0	$13.75^{a} \pm 2.22$	$13.5^{a} \pm 2.08$	$7.75^{a} \pm 1.50$
T1	$12.00^{a} \pm 2.94$	$11.00^{a} \pm 1.63$	$7.00^{a} \pm 2.16$
T2	$13.25^{a} \pm 2.22$	$11.75^{a} \pm 1.70$	$7.75^{a} \pm 3.09$
T3	$15.25^{a} \pm 1.89$	$4.00^{b} \pm 1.41$	$10.50^{a} \pm 1.73$
T4	$13.75^{a} \pm 2.63$	$4.50^{b} \pm 2.08$	$6.75^{a} \pm 2.50$

a, b: Means within a column with different superscripts differ significantly ($p < 0.05$). T0: negative control; T1: positive control or infected with *E. tenella*; T2: 5ml/kg feed of VCO; T3: 10ml/kg feed of VCO; T4: 20ml/kg feed of VCO.

Table 4. The result of monocyte count (%) of the VCO effect in chicken infected with *E. tenella*

Treatment	Day after infection		
	0	3	6
T0	$5.50^{ab} \pm 1.91$	$5.50^{b} \pm 1.29$	$7.75^{a} \pm 1.50$
T1	$2.00^{c} \pm 0.82$	$8.25^{a} \pm 2.06$	$7.00^{a} \pm 2.16$
T2	$6.50^{ab} \pm 2.08$	$4.75^{b} \pm 1.26$	$7.75^{a} \pm 3.09$
T3	$7.75^{a} \pm 2.50$	$4.00^{b} \pm 1.41$	$10.50^{a} \pm 1.73$
T4	$4.50^{bc} \pm 1.00$	$4.50^{b} \pm 2.08$	$6.75^{a} \pm 2.50$

a, b, c: Means within a column with different superscripts differ significantly ($p < 0.05$). T0: negative control, T1: positive control or infected with *E. tenella*, T2: 5 ml/kg feed of the VCO, T3: 10 ml/kg feed of the VCO, T4: 20ml/kg feed of the VCO.

Table 5. The results of the lymphocyte count of the VCO effect in chicken infected with *E. tenella*

Treatment	Day after infection		
	0	3	6
T0	$72.00^{b} \pm 3.74$	$77.00^{a} \pm 2.70$	$79.75^{b} \pm 0.50$
T1	$80.00^{a} \pm 5.71$	$73.75^{a} \pm 2.87$	$73.75^{c} \pm 2.87$
T2	$75.50^{ab} \pm 3.78$	$78.50^{a} \pm 2.08$	$82.00^{b} \pm 2.45$
T3	$72.50^{b} \pm 4.04$	$76.75^{a} \pm 4.35$	$77.25^{bc} \pm 5.73$
T4	$77.75^{ab} \pm 0.16$	$75.25^{a} \pm 4.19$	$88.75^{a} \pm 5.93$

a,b,c: Means within a column with different superscripts differ significantly ($p < 0.05$). T0: negative control, T1: positive control or infected with *E. tenella*, T2: 5 ml/kg feed of VCO, T3: 10 ml/kg feed of VCO, T4: 20 ml/kg feed of VCO.

DISCUSSION

The average basophil count in each group was about zero to two percent or was still within the normal basophil range of one to four percent (Current et al., 1983), which proved that VCO was able to affect the amount of basophil. Basophil played the weakest role in the immune system (Tizard, 2013; Eberle and Voehringer, 2016), which is rarely found in the poultry blood under normal conditions. That is the reason for the low percentage of basophil in the total blood leukocytes.

The result also showed that the basophil increased on the third day after infection, possibly due to the fact that the basophil was stimulated at the inflammation site on the caecal epithelial caused by *E. tenella*. Basophil contains serotonin, heparin, and histamine that stimulate blood flow to the site of inflammation (Bijanti et al., 2010). Otherwise, the basophil decreased on the sixth day after infection, possibly because VCO acts as an anti-inflammation substance (Sharifi-Rad et al., 2017). Virgin coconut oil has anti-inflammatory, moderate analgesic, and antipyretic properties (Intahphuak et al., 2010). It had been shown that fatty acids such as oleic and stearic acids in VCO attenuated the activity of polymorph nuclear

leukocytes, which led to the suppression of inflammatory processes. It was suggested that the proportion of fatty acids integrated into membrane phospholipids affect membrane fluidity, which in turn could influence cell function (Zakaria et al., 2011). VCO had monolaurin and monocaprin substance, a monoglyceride substance that was used as anti-microbe, anti-viral, antibacterial, and antiprotozoal because they can dissolve the lipid-coated wall of the microbes, causing the cells of the microbes to rupture and die, so the amount of the parasites in the tissues and the amount of basophil would reduce (Arlee et al., 2013).

The data showed a significantly different ($p < 0.05$) eosinophil counts. The data showed that the T3 and T4 group had the highest amount of increasing number of eosinophil on the third day after infection. Increasing numbers of eosinophils might be due to the VCO, which could help stimulate the immune system of the chicken. Even though eosinophil is most active during helminth parasite infection, some studies stated that the coccidian parasite could also lead to eosinophilia (Nutman, 2007). On the sixth day after infection, the most decreasing amount of eosinophil was observed in the T3 and T4 group. This might be because the VCO acted as an antiprotozoal and anti-inflammation, so the number of parasites in the tissues and the amount of eosinophil would decrease.

The normal range of eosinophil in the blood is about 2-8% of the total amount of leukocytes, so the number of eosinophil in the present research was still in the normal range. Eosinophil neutralize inflammatory factor released by mast cells and basophils in type 1 hypersensitivity reaction (Tizard, 2013). Eosinophil acts as a regulator of parasite infection by attaching to the parasite and releasing toxic substances to the parasite (Wen and Rothenberg, 2016). Eosinophil regulates the allergic reaction and acute inflammation, which can stimulate tissue damages (Jain, 1993).

It was known that eosinophils interact with homocytotropic antibodies (IgE and IgG), mast cells, and basophils. The antibody and T lymphocytes provided specificity for the reaction, and the IgE on mast cells attracted eosinophils to modulate the inflammatory reaction. The relative amounts of tissue IgE, extractable histamine, and eosinophil suggested that these components form the immune system that was most pronounced on body surfaces, immunologically mediated, often parasitic related and frequently associated with eosinophilia (Ross et al., 1999).

The data in the table 3 indicate that there was a significant number of heterophilic differences ($p < 0.05$) between each day after infection, although there were still decreasing numbers of heterophil on the third and sixth days after infection. The results shown in the table 3 also announce that the average amount of heterophil tended to decrease constantly. A decrease in the amount of heterophil might be because heterophil has the opposite effect on lymphocytes called the H/ L ratio and this ratio is influenced by diseases and infection (or the stress hormones produced by infection) (Davis et al., 2008). It could also happen because VCO stimulated the host cell-mediated immune system, which was done by lymphocyte T.

Heterophiles could survive in tissues for one to two days. In the present study, however, the heterophiles decreased steadily for six days, possibly because VCO constantly helps reduce the inflammatory factors as was given to the chickens daily. Heterophile has most of the lysosomal enzymes, which are proteolytic enzymes used to digest bacteria and foreign protein materials (Guyton and Hall, 2006). Heterophiles find, digest, and kill foreign bodies and also acts as the first line of defense (Ganong and Ganong, 1995).

In table 3, the number of heterophile (8.33%-11.67%) was considered below the normal range (20.9% or 25%-30%) and reported as heteropenia. Heteropenia could be observed in the chicken infected with *E. coli* after three and six hours. Heteropenia or heterophilia in chickens could occurred with inflammation or infection, stress and sometimes neoplasia (DeRosa et al., 1992).

The data in table 4 indicate a significant difference in monocyte counts, although the T4 group had the lowest number of monocytes on the sixth day after infection, as VCO might reduce the oocyst in the chicken, which would lead to a decrease in the inflammatory factor. If the inflammatory factor is reduced, it also decreases the migration of monocytes to the caecal tissue. The reduction in the monocytes could indicate the healing process. The administration of VCO increased the secretion of thyroid hormone, which led to an increase in the metabolism process. If the metabolism increases, the cell function will be more efficient, which helps to protect the body from the bad condition and also accelerate the healing process (Boateng et al., 2016).

Monocytes are produced in the bone marrow about three to eight percent of the total leukocytes in the blood. Monocytes in poultry are the largest leukocytes with various forms from rounded to amoeboid shape. Compared to lymphocytes, the chromatin granules of the

monocyte nucleus are less accumulated. Monocytes indicated phagocytic activity and migration into the tissues to become macrophages (Bijanti et al., 2010). Apart from the macrophage, monocytes are important to maintain the immune response by releasing glycoprotein regulators/monokines such as interferon, interleukin-l, hormone-like AMP (Adenosine Monophosphate), and active pharmacological substances such as prostaglandines and leukotrienes (Tizard, 1982).

The data in table 5 reveal significantly different ($p < 0.05$) lymphocyte counts, but the group treated with VCO indicated a more constant increase in lymphocyte count than the control negative group, and the T4 group had the most lymphocytes counts. It was known that palmitate and myristate acid in VCO are the phospholipid component of T lymphocytes. Therefore, VCO could stimulate lymphocytes and produce an antibody in the chickens (Gordon, 2003).

The T lymphocyte plays an important role in stimulating the immune system against certain diseases and stress factors. The T lymphocyte would react directly to antigen presented to the cell surface by an Antigen-Presenting Cell (APC). The Th-CD4 interaction served to maintain the Th-APC binding intact during the specific antigen activation (Hussain et al., 2004). Lymphocyte Th would activate macrophages as a cellular immune response against infection with the intracellular pathogen (Gordon, 2003).

The result in table 5 shows that the T4 group had the highest lymphocyte counts, although the T3 had the lowest lymphocyte counts and the most stable increase in the lymphocyte counts. Possibly because VCO reduced the oocysts, so that the lymphocyte counts reduced. The stable increase could indicate the VCO function as an immunostimulant. The percentage range of lymphocyte count for T0-T3 was 75.5% to 82% or was still in the normal range of lymphocytes (24%-84% of total leukocytes in the blood), but the T4 group on the sixth day after the infection indicated that the lymphocytes count was more than the normal range, possibly because the stress effect of the chicken or the 20 ml/kg feed dose of VCO had too much influence on the lymphocyte T production.

Eimeria tenella infection in chicken could cause the lymphocyte counts to reach 87% or exceed the normal range. The increase in the lymphocyte count could attributed to the effect of the inflammation of the caeca-intestine (Patra et al., 2010). Chronic antigenic stimulation could lead to a greatly expanded circulating lymphocyte pool since the primary functions of the lymphocyte are immune response, humoral antibody production, and cell-mediated immunity (Jones, 1999). Cell-mediated immunity plays an important role in protecting the chicken from coccidial infection.

There is increasing evidence that cell-mediated immunity plays a major role in resistance to infection, as T lymphocytes appear to respond to coccidial infection by both cytokine production and a direct cytotoxic attack on infected cells (Lillehoj and Trout, 1996; Yun et al., 2000).

CONCLUSION

The research result proved that adding Virgin coconut oil could improve the different leukocyte counts of chicken infected with *E. tenella*. The virgin coconut oil at the dose of 10 ml/kg feed as a dietary supplementation and 20 ml/kg feed had the most significant result in improving the different leukocytes counts of the chicken infected with *E. tenella*. So, dietary ingestion of efficient vegetable oil especially Virgin coconut oil, regardless of efficient farming, could be useful in combating some parasitic diseases.

DECLARATION

Authors' contribution

All authors of manuscript; Z. Sheila Faradilla, Muchammad Yunus, and H.A. Hermadi had similar and continuous attempts *in in vivo* and *in vitro* experiments of present study.

Competing interests

The authors have declared there was no conflict of interest.

Acknowledgments

The authors thank the staff of the Faculty of Veterinary Medicine, Universitas Airlangga for supports and facilities.

REFERENCES

Abdul Mohymen N, Qader A, and Ad'hiah A (2014). Molecular Typing by Polymerase Chain Reaction Sequence Specific Primers (PCR- SSP) of Human Leukocyte Class I and Class II Alleles in a Sample of Iraqi Visceral Leishmaniasis Patients. International Journal of Advanced Biological and Biomedical Research, 2(8): 2412-2415. Available at: http://www.ijabbr.com/article_8440.html

Akinnuga AM, Jeje SO, Bamidele O, Amaku EE, Otogo FO, and Sunday VE (2014). Virgin coconut oil: Remedial effects on renal dysfunction in diabetic rats. Physiology Journal, 2014: 495926. DOI: https://doi.org/10.1155/2014/495926

Alfaro DM, Silva AVF, Borges SA, Maiorka FA, Vargas S, and

Santin E (2007). Use of *Yucca schidigera* extract in broiler diets and its effects on performance results obtained with different coccidiosis control methods. Journal of Applied Poultry Research, 16: 248–254. DOI: https://doi.org/10.1093/japr/16.2.248

Arlee R, Suanphairoch S, and Pakdeechanuan P (2013). Differences in chemical components and antioxidant-related substances in virgin coconut oil from coconut hybrids and their parents. International Food Research Journal, 20(5): 2103-2109. Available at: http://www.ifrj.upm.edu.my/20%20(05)%202013/9%20IFRJ%2020%20(05)%202013%20Pakdeechanuan%20182.pdf

Attia YA, Al-Harthi MA, and Abo El-Maaty HM (2020). The Effects of different oil sources on performance, digestive enzymes, carcass traits, biochemical, immunological, antioxidant, and morphometric responses of broiler Chicks. Frontiers in Veterinary Science, 7: 181. DOI: https://doi.org/10.3389/fvets.2020.00181

Bachene MS, Temim S, Ainbaziz H and Bachene A (2019). Prevalence of rabbit coccidia in Medea Province, Algeria. World Veterinary Journal, 9(2): 123-128. Available at: http://wvj.science-line.com/attachments/article/57/WVJ%209(2)%20123-128,%20June%2025,%202019.pdf

Berezin VE, Bogoyavlenskyi AP, Khudiakova SS, Alexuk PG, Omirtaeva ES, Zaitceva IA, Tustikbaeva GB, Barfield RC, and Fetterer RH (2010). Immunostimulatory complexes containing *Eimeria tenella* antigens and low toxicity plant saponins induce antibody response and provide protection from a challenge in broiler chickens. Veterinary Parasitology, 167: 28-35. DOI: https://doi.org/10.1016/j.vetpar.2009.09.045

Bijanti R, GandulAtikYuliani M, Wahjuni RS, and Utomo RB (2010). Veterinary Clinical Pathology Textbook. Airlangga University Press. Pp.53-107. Available at: https://www.wiley.com/en-us/Fundamentals+of+Veterinary+Clinical+Pathology%2C+2nd+Edition-p-9780813800769

Boateng L, Ansong R, Owusu W, and Steiner-Asiedu M (2016). Coconut oil and palm oil's role in nutrition, health, and national development: A review. Ghana Medical Journal, 50: 189-196. PMID: 27752194

Chapman HD (2008). Coccidiosis in the turkey. Avian Pathology, 37: 205–223. DOI: https://doi.org/10.1080/03079450802050689

Chengat Prakashbabu B, Thenmozhi V, Limon G, Kundu K, Kumar S, Garg R, Clark EL, Srinivasa Rao AS, Raj DG et al. (2017). *Eimeria* species occurrence varies between geographic regions and poultry production systems and may influence parasite genetic diversity. Veterinary Parasitology, 233: 62–72. DOI: https://doi.org/10.1016/j.vetpar.2016.12.003

Current WL, Reese NC, Ernst JV, Bailey WS, Heyman MB, and Weinstein WM (1983). Human cryptosporidiosis in immunocompetent and immunodeficient persons: studies of an outbreak and experimental transmission. The New England Journal of Medicine, 308: 1252–1257. DOI: https://doi.org/10.1056/nejm198305263082102

Davis AK, Maney DL, and Maerz JC (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology, 22: 760–772. DOI: https://doi.org/10.1111/j.1365-2435.2008.01467.x

Debbou-Iouknane N, Benbarek H, and Ayad A (2018). Prevalence and aetiology of coccidiosis in broiler chickens in Bejaia province, Algeria. The Onderstepoort Journal of Veterinary Research, 85(1): e1–e6. DOI: https://doi.org/10.4102/ojvr.v85i1.1590

Dubey JP (2018). A review of coccidiosis in South American camelids. Parasitology Research, 117(7): 1999–2013. DOI: https://doi.org/10.1007/s00436-018-5890-y

DeRosa M, Ficken MD, and Barnes HJ (1992). Acute airsacculitis in untreated and cyclophosphamide-pretreated broiler chickens inoculated with Escherichia coli or Escherichia coli cell-free culture filtrate. Veterinary Pathology, 29: 68–78. DOI: https://doi.org/10.1177/030098589202900109

Eberle JU, and Voehringer (2016). Role of basophils in protective immunity to parasitic infections. Seminars in Immunopathology, 38: 605–613. DOI: https://doi.org/10.1007/s00281-016-0563-3

Fatoba AJ, and Adeleke MA (2018). Diagnosis and control of chicken coccidiosis: a recent update. Journal of Parasitic Diseases, 42: 483–493. DOI: https://doi.org/10.1007/s12639-018-1048-1

Ganong WF, and Ganong W (1995). Review of medical physiology. Appleton and Lange Norwalk, CT, UK. Pp.70-83. Available at: https://emergencypedia.files.wordpress.com/2013/04/ganong-pdf.pdf

Gordon S (2003). Alternative activation of macrophages. Nature Reviews Immunology, 3: 23. DOI: https://wwww.doi.org/10.1038/nri978

Guyton AC, and Hall JE (2006). Textbook of medical physiology. 11th edition. WB Saunders Company, Philadelphia. Pp.1002-1010. Availble at: https://www.amazon.com/Textbook-Medical-Physiology-11th-Eleventh/dp/B002NAT0F6

Habibi H, Firouzi S, Nili H, Razavi M, Asadi L, and Daneshi S (2016). Anticoccidial effects of herbal extracts on *Eimeria tenella* infection in broiler chickens: in vitro and in vivo study. Journal of Parasitic Diseases, 40(2): 401–407. DOI: https://doi.org/10.1007/s12639-014-0517-4

Hussain MI, Khan SA, Chaudhary ZI, Aslam A, Ashraf K, and Rai MF (2004). Effect of organic and inorganic selenium with and without vitamin E on the immune system of broilers. Pakistan Veterinary Journal, 24: 1–4. Availble at: http://pvj.com.pk/pdf-files/24_1/1-4.pdf

Intahphuak S, Khonsung P, and Panthong A (2010). Anti-inflammatory, analgesic, and antipyretic activities of virgin coconut oil. Pharmaceutical Biology, 48: 151–157. DOI: https://doi.org/10.3109/13880200903062614

Jain NC (1993). Essentials of veterinary hematology copyrights by Lea and Febiger, Philadelphia. Pp.1002-1036. Available at: https://www.semanticscholar.org/paper/Essentials-of-Veterinary-Hematology-Jain/d6fb81de5398593260f72bd9e665c10c678c53c4?p2df

Jones MP (1999). Avian clinical pathology. Veterinary Clinics of North America: Exotic Animal Practice, 2: 663–687. DOI:

https://doi.org/10.1016/s1094-9194(17)30115-9

Lillehoj HS, and Trout JM (1996). Avian gut-associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. Clinical microbiology reviews, 9: 349–360. PMID: 8809465

Long PL (1968). The pathogenic effects of *Eimeria praecox* and *E. acervulina* in the chicken. Parasitology, 58: 691–700. DOI: https://doi.org/10.1017/s0031182000028997

Macer D (2019). Ethical poultry and the bioethics of poultry production. The Journal of Poultry Science, 56(2): 79–83. DOI: https://doi.org/10.2141/jpsa.0180074

Muazu A, Masdooq AA, Ngbede J, Salihu AE, Haruna G, Habu AK, Sati MN, and Jamilu H (2008). Prevalence and identification of species of *Eimeria* causing coccidiosis in poultry within Vom, Plateau State, Nigeria. International Journal of Poultry Science, 7: 917–918. DOI: https://dx.doi.org/10.3923/ijps.2008.917.918

Nutman TB (2007). Evaluation and differential diagnosis of marked, persistent eosinophilia.

Immunology and Allergy Clinics of North America, 27: 529–549. DOI: https://doi.org/10.1016/j.iac.2007.07.008

Patra G, Ali MA, Chanu KV, Jonathan L, Joy LK, Prava M, Ravindran R, Das G, and Devi LI (2010). PCR based diagnosis of *Eimeria tenella* infection in broiler chicken. International Journal of Poultry Science, 9: 813–818. DOI: 10.3923/ijps.2010.813.818

Pop LM, Varga E, and Coroian M (2019). Efficacy of a commercial herbal formula in chicken experimental coccidiosis. Parasites Vectors, 12: 343. DOI: https://doi.org/10.1186/s13071-019-3595-4

Roeslan BO (1996). Karakteristik Streptococcus mutans penyebab karies gigi (Characteristic *Streptococcus mutans*). Majalah Ilmiah Kedokteran Gigi FKG Usakti, 10: 112–113. Available at: https://scholar.google.co.id/scholar?oi=bibs&cluster=7414454324532419892&btnI=1&hl=en

Ross TM, Oran AE, and Cullen BR (1999). Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. CurrentBiology, 9: 613–621. DOI: 10.1016/s0960-9822(99)80283-8

Sharifi-Rad J, Sureda A, Tenore GC, Daglia M, Sharifi-Rad M, Valussi M, Tundis R, Sharifi-Rad M, Loizzo MR, Ademiluyi AO et al. (2017). Biological activities of essential oils: from plant chemoecology to traditional healing systems. Molecules (Basel, Switzerland), 22(1): 70. DOI: https://doi.org/10.3390/molecules22010070

Tan GH, and Long K (2012). A preliminary study of the anticoccidial activity of medium-chain fatty acids (MCFA) and their corresponding monoglycerides on broiler chicken coccidiosis International Journal of Biotechnology for Wellness Industries, 1: 134–141. Available at: https://www.lifescienceglobal.com/pms/index.php/ijbwi/article/download/36/101

Tipu MA, Pasha TN, and Ali Z (2002). Comparative Efficacy of Salinomycin Sodium and Neem Fruit (Azadirachta Indica). International Journal of Poultry Science1, Pp. 91–93. DOI: https://doi.org/10.3923/ijps.2002.91.93

Tizard I (1982). An introduction to veterinary immunology. Immunology and Allergy Clinics of North America, 27: 529-549. DOI: https://doi.org/10.1111/j.1365-3164.2009.00733.x

Tizard IR (2013). Veterinary Immunology-E-Book. Elsevier Health Sciences. Pp.110-125. Available at: https://www.elsevier.com/books/veterinary-immunology/tizard/978-1-4557-0362-3

Wen T, and Rothenberg ME (2016). The regulatory function of eosinophils. Microbiology Spectrum, 4(5): 10. DOI: https://doi.org/10.1128/microbiolspec.MCHD-0020-2015

Yun CH, Lillehoj HS, and Lillehoj EP (2000). Intestinal immune responses to coccidiosis. Developmental and Comparative Immunology, 24: 303–324. DOI: https://doi.org/10.1016/s0145-305x(99)00080-4

Zakaria ZA, Somchit MN, Jais AMM, Teh LK, Salleh MZ, and Long K (2011). In vivo antinociceptive and anti-inflammatory activities of dried and fermented processed virgin coconut oil. Medical Principles and Practice, 20: 231–236. DOI: https://doi.org/10.1159/000323756

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 623-630, December 25, 2020

Research Paper, PII: S2322455X2000071-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.71

Characteristics of Carcass Traits and Meat Quality of Broiler Chickens Reared under Conventional and Free-range Systems

Peymaneh Davoodi and Alireza Ehsani*

Department of Animal Science, Tarbiat Modares University, Tehran, PO Box 14115-336, Iran.
*Corresponding author's E-mail: alireza.ehsani@modares.ac.ir ; ORCID: 0000-0001-6933-3469

Received: 01 Nov. 2020
Accepted: 14 Dec. 2020

ABSTRACT

Alternative chicken production systems have become popular in recent years due to animal welfare criteria and consumer's perceptions. General beliefs express that the meat quality of chicken reared under free-range systems is better than that of chickens under conventional production conditions. The aim of this study was to compare the meat quality and carcass traits of chickens raised in conventional and free-range systems. Either conventional or free-range systems used meat-type Hubbard JA57 birds with a slaughter age of approximately 78 days. For assessing carcass traits and meat quality, six male chickens were selected from each system. The meat quality parameters, pH at 45 minutes, ultimate pH, color coordinates, drip loss, cooking loss, and water-holding capacity were measured. Furthermore, proximate parameters, such as crude protein, total fat, and crude ash were determined. There were no significant differences in main carcass yield and breast muscles between chickens reared in two systems, however, color values dramatically were influenced by rearing systems. Breast muscle samples from birds reared under the conventional system had a smaller hue angle and saturation value than those from the free-range birds. Moreover, the drip loss parameter was significantly higher in free-range chickens. The ash and protein contents of breast muscles were similar although raw breast meat from free-range birds had significantly lower fat content. The results prove that a free-range rearing system can modify the appearance, color values, and fat content of chicken meat and it can be a part of the interests of meat production consumers.

Keywords: Broiler chickens, Free-range, Hubbard JA57, Intensive rearing system, Meat quality

INTRODUCTION

Chickens were primarily raised on family farms outdoor until around the 1950s (Alvarado et al., 2005). Since then the remarkable growth of chicken meat production commenced when the modern poultry industry began. This has led to developing intensive rearing systems, ensuring highest effectiveness and profitability of production (Bogosavljevi-Boskovic et al., 2012). However, intensive systems and rapid growth can cause animal stress, resulting in undesirable physiological and behavioral responses which lead to poor performance and meat quality (Xing et al., 2019) and occurrence of idiopathic myopathies, white striping, wooden breast, as well as pale, soft and exudative (PSE)-like meat (Ishamri and Seon-Tea, 2017). In contrast, free-range system can decrease stress conditions and allow better chicken welfare without causing any environmental confinement on broilers (Santos et al., 2005; da Silva et al., 2017). The poultry meat quality, in general, is an extremely complex concept that can be evaluated from different aspects because it is dependent on numerous factors, including genotype, sex, age, diet, density, environment and also rearing system (Berg, 2001; Brown et al., 2008; Miele, 2011; da Silva et al., 2017). In this respect, over the last decades, poultry meat production has paid particular attention to animal welfare, high-quality food safety standards, and different rearing systems. So, the competitiveness in this market has been radically changed into equally both price and quality competitiveness (Berg, 2001; Yeung and Morris, 2001; Bogosavljevic-Boskovic et al., 2010; Saleh et al., 2015).

From the prevailing viewpoint, broilers should have not only high slaughter yields and desirable carcass conformation scores, but also proper aesthetic, nutritional, and healthy characteristics. Hereupon, the chemical composition of chicken meat is another essential factor of broiler meat quality (Berg, 2001; Yeung and Morris, 2001; Castellini et al., 2008; Wang et al., 2009; Miele, 2011; Saleh et al., 2015; Srednicka-Tober et al., 2016). According to previous studies, broilers that had outdoor access showed better meat quality considering chemical

To cite this paper: Davoodi P and Ehsani A (2020). Characteristics of Carcass Traits and Meat Quality of Broiler Chickens Reared under Conventional and Free-range Systems. *J. World Poult. Res.*, 10 (4): 623-630. DOI: https://dx.doi.org/10.36380/jwpr.2020.71

composition and healthy characteristics of produced meat (Berg, 2001; Castellini et al., 2008; Miele, 2011; Saleh et al., 2015; Srednicka-Tober et al., 2016). Castellini et al. (2002) and Lin et al. (2014) also claimed that chemical contents in the chicken meat produced in free-range were better than those from other conventional systems, also meat sensory scores and overall acceptability in the free-range group were higher than those in other systems. In addition, high protein content, and low fat in meat of free-range chickens offers a healthier diet choice (Lin et al., 2014). Given that the consequence of rearing systems can differ by fattening length, the density of the flock, time to access free range, climatic factors, seasons and breeds, the results reported in previous studies are often quite variable (Bogosavljevi-Boskovic et al., 2012; Srednicka-Tober et al., 2016). Some authors have reported no significant effect of rearing system on carcass yields and meat quality traits but others have obtained statistically significant differences (Wang et al., 2009). Therefore, the objective of the current study was to evaluate the growth performance, carcass characteristics, and meat quality of Hubbard JA57 strain (known for free-range rearing) in both conventional and free-range systems in Iran condition.

MATERIALS AND METHODS

Ethics approval

The Ethics Committee on the Use of Animals of Tarbiat Modares University, Iran approved all the experiments and protocols used in this study (14-67626). All experimental protocols were applied in accordance with relevant legislation and recommendations by this committee.

Experimental schedule and sample collection

This trial was carried out at the Poultry Experimental Farm in Tarbiat Modares University, Iran from September to November 2017. The test material used in this study included a total of 82 fertile eggs of slow-growing Hubbard JA57 strain imported from Denmark. The eggs were placed in an incubator (Victoria Como, Italy) with the broad end pointing upwards and proper temperature and humidity. At the end of 18 days of incubation, the eggs were transferred into individual pedigree hatch bags sewn with a net fabric in order that chicks could be traced back to their initial egg weights. The hatch was pulled at day 21.5 with hatchability of 91%, then all 74 day-old chicks generated were identified with left leg tags using plastic cable ties and housed in a standard conventional system until the end of week 5 and then divided randomly into two groups each with 36 birds, one was reared in the same house and the other was transferred to the free-range system until the end of the rearing period of 11 weeks. Birds were provided with the same starter and finisher diets (Table 1), formulated based on the chemical composition of ingredients and broiler nutritional requirements according to NRC (NRC, 1994). The summary of the rearing systems conditions is given in Table 2. At the end of the rearing period of 11 weeks, 12 male broilers (6 from each rearing system) with a target weight range of 3.2 to 3.5 kg were randomly chosen to provide materials for analysis and sent to the chicken slaughterhouse related to the Department of Animal Sciences of Tarbiat Modares University, Iran to assess carcass characteristics and meat quality. After fasting for 12 hours before slaughter, all birds were weighed individually, slaughtered, and processed using standard commercial practices. All of them were used for physical, chemical, raw and cooked product analyses. The organ weights (liver, spleen, gizzard, heart, bursa of Fabricius) were recorded.

Table 1. Ingredients of starter and finisher diets and Dry matter based proximate chemical composition of diets.

Item	Starter (1-4 weeks)	Finisher (5-11 weeks)
Ingredient		
Maize	59.78	66.00
Soybean meal	28.52	22.00
Soybean oil	4.50	3.80
Wheat bran	4.56	5.92
Dicalcium phosphate	1.05	0.60
Limestone powder	0.90	1.05
Mineral and Vitamin Mix	0.34	0.34
Salt	0.10	0.10
Lysine	0.10	0.13
Methionine	0.15	0.06
Threonine	0.00	0.06
Chemical composition		
Metabolizable energy (kcal/kg)	3100	3150
Crude protein (%)	23.00	20.00
Crude fiber (%)	2.44	2.60
Ether extract (%)	7.16	6.20
Lysine (%)	1.12	1.00
Methionine (%)	0.50	0.39
Calcium (%)	1.00	0.90

Table 2. Summary of rearing conditions for chickens raised under two different systems.

Items	Conventional rearing system	Free-range rearing system
Outdoor access	No access	Access after week 5
Diet	*Ad libitum* regular diet with no animal ingredient	*Ad libitum* regular diet with no animal ingredient + access to pasture
Water	*Ad libitum*	*Ad libitum*
Vaccine program	Regular vaccination against Newcastle	Regular vaccination against Newcastle
Antibiotic	No antibiotic	No antibiotic
Slaughter age (day)	78	78

Measurements and evaluations

The meat quality variables, carcass component yield, initial and ultimate pH, color coordinates (Lightness, L*; redness, a*; yellowness, b*), Hue angle, saturation, drip loss, and water-holding capacity were measured. Also, cooked breast samples were evaluated for cooking yield (Mikulski et al., 2011).

Carcass component yield

The carcass weight was obtained after removing the head, neck, and shanks. Then, the main commercial segments (e.g., warm carcass, breast, and leg) and marginal parts (e.g., wings, abdominal fat, spleen, testicles, heart, and gallbladder) were weighed. Finally, the values were expressed as a percentage of carcass weight (Santos et al., 2005; Comert et al., 2016).

pH

The initial pH (45 min after slaughtering) and ultimate pH (pH in 24 h after slaughtering) were measured on raw homogenized breast muscles of six chickens from each group with three replications, in the same procedure. Approximately 2.5 g of meat was removed from the center of each pectoral major muscle, minced by mortar and pestle, and suspended in 25 mL distilled water then centrifuged for 5 min at 6000 rpm. Measurement of pH was performed using a digital pH meter equipped with a sensitive electrode, the device was calibrated before measurement at pH 4.0 and 7.0 buffer solutions (Choo et al., 2014).

Cooking loss

Cooking loss was determined by weighing meat before and after cooking. Meats were enveloped in an aluminum foil and cooked in an electric oven at 100 °C for 15 min, then samples were removed from the oven and left to cool at room temperature (Lin et al., 2014).

Drip loss

Drip loss was measured by keeping samples suspended in covered plastic bags on plastic racks for 48 h at 2 °C and calculated as a percentage of weight loss during storage (Funaro et al., 2014).

Water-holding capacity

Water-holding capacity was determined by the filter paper press method to obtain expressible meat juice. A 1000 mg raw meat sample was placed between several pieces of filter paper with 11 cm diameter and pressed for 5 min. Expressed juice of meat was defined as the loss in weight after pressing and expressed as a percentage of the initial weight of the raw meat sample (Wierbicki and Deatherage, 1958; Lee, 1995).

Color

The color profile of lightness, L*; redness, a*; yellowness, b* was measured by a reflectance colorimeter (ColorFlex EZ Spectrophotometer, USA) in triplicate on raw breast meat. The device was calibrated with black and white standards before meat color determination. Hue angle and saturation index were measured using formulas h_{ab} = arctan (b*/a*) and S = ([a^{*2} + b^{*2}]^{½})/ L*, respectively (da Silva et al., 2017).

Proximate analysis

Protein, fat, and ash content of raw breast were each independently measured with three replications from each of 6 broilers from free-range and 6 broilers from standard rearing system. Samples for proximate analysis were frozen until analyzed at the laboratory of the Animal Science Faculty of Tarbiat Modares University. Protein, fat, and ash content was measured following the AOAC methodology (Lee, 1995).

Statistical analysis

The R 4.0.2 software was used for statistical analysis. Pairwise treatment differences between carcass traits obtained from two different rearing system were determined with the Student t-test and any differences were considered significant at $p < 0.05$, for this reason, data were summarized as mean ± standard deviation and analyzed by one-way analysis of variance taking the rearing system as the main effect.

RESULTS

The comparison of carcass traits between the two rearing systems is reported in Table 3. There were no significant effects of rearing systems on the main carcass parameters measured. The results of physical and chemical meat characteristics comparison between conventional and the

free-range system are presented in Table 4. The initial pH of the breast meat from free-range broilers was slightly lower than conventional even though the ultimate pH of the breast meat became higher in free-range than conventional birds. These differences were not statistically significant but the difference of initial and ultimate pH values in free-range birds showed significant change ($p = 0.004$).

Table 3. Carcass characteristics of chickens reared under conventional and free-range systems.

Trait	Conventional system	Free-range system	t statistics	p-value
Live Weight (g)	3548.33	3371.67	-1.279	0.229
Carcass Weight (g)	2770.83	2560.00	-1.722	0.115
Drop Carcass (g)	777.50	811.67	0.758	0.465
Tight (%)	25.56	25.31	-0.424	0.680
Breast (%)	26.87	27.29	0.509	0.621
Wings (%)	11.63	10.97	-1.353	0.205
Legs (%)	4.57	4.74	1.197	0.258
Liver (%)	2.10	2.22	0.899	0.389
Abdominal fat (%)	2.50	2.53	0.114	0.911
Gizzard (%)	1.46	2.45	2.002	0.096
Testicle (%)	0.15	0.07	-2.314	0.061
Burse (%)	0.13	0.18	1.299	0.222
Gallbladder (%)	0.15	0.10	-1.908	0.085
Spleen (%)	0.19	0.22	1.360	0.203
Heart (%)	0.63	0.60	-1.244	0.241

Each group contained 36 chickens. *Statistically significant at $p < 0.05$

Table 4. Chicken carcass quality traits in conventional and free-range rearing systems.

Meat quality traits	Conventional system	Free-range system	t-statistics	p-value
pH measures				
Initial pH	5.81 ± 0.11	5.78 ± 0.15	-0.379	0.713
Ultimate pH	5.77 ± 0.14	5.83 ± 0.09	1.450	0.178
pH difference	-0.04 ± 0.10	0.05 ± 0.10	3.727	0.004*
Meat quality metrics				
Cooking loss	29.33 ± 2.45	28.10 ± 1.70	-1.259	0.237
Drip loss	3.24 ± 1.22	3.70 ± 1.60	2.384	0.038*
Water holding capacity	53.03 ± 2.32	55.26 ± 1.80	1.92	0.08
Color parameters				
Lightness	59.92 ± 0.33	52.00 ± 2.70	-7.008	0.000*
Redness	7.29 ± 1.79	3.40 ± 1.30	-4.309	0.002*
Yellowness	11.01 ± 2.04	13.20 ± 0.90	2.461	0.034*
Hue angle	56.40 ± 10.03	75.60 ± 4.80	4.233	0.002*
Saturation	13.40 ± 1.29	13.70 ± 1.00	4.902	0.001*

Data are expressed as mean ± standard deviation. Each group contained six chickens. *Statistically significant at $p < 0.05$.

Table 5. Proximate analysis of raw breast meat from conventional and free-range broilers.

Proximate analysis	Conventional system	Free-range system	t-statistics	p-value
Protein, %	23.85 ± 0.41	24.20 ± 0.54	1.23	0.24
Fat, %	4.01 ± 0.40	3.18 ± 0.40	-3.57	0.005*
Ash, %	3.03 ± 0.21	3.05 ± 0.28	-0.12	0.90

Data are expressed as mean ± standard deviation. Each group contained six chickens. *Statistically significant at $p < 0.05$

The effect of the two rearing systems on meat quality did not represent a significant difference in terms of the cooking loss and water holding capacity parameters, however, the drip loss parameter was affected by rearing systems significantly ($p = 0.038$). All the color coordinates L*, a*, b*, hue angle, and saturation value also showed extremely significant differences between the rearing systems. Breast muscle from free-range birds revealed significantly lower L* and a* values making it paler in comparison to conventional breast muscle. The smallest difference was seen in the b* coordinate with the breast muscle from the free-range birds which was significantly higher than conventionally reared birds, so breast meat in free-range was more yellow ($p = 0.03$) than conventional.

The result of the proximate analysis of the raw breast meat produced from chickens reared under conventional and the free-range system is reported in Table 5. Raw breast meat from free-range birds had significantly ($p = 0.005$) lower fat content in comparison to broilers reared conventional system, however, no significant differences ($p > 0.05$) was observed for protein and ash content of the raw breast meat.

DISCUSSION

A growing knowledge of human health, food safety, and animal welfare interests have led to the great transformation in animal rearing systems and particularly free-range product markets all over the world. Chicken meat produced in alternative systems, such as free-range or organic, are part of this orientation. It is specified that slaughtering age, genetic breeds (fast and slow-growing), physical activity, and pasture intake are key factors in chicken meat quality.

In this research, no significant differences were observed in yield of carcass parts. The result has no consistency with the findings of Mikulski et al. (2011) who reported the significant influence of rearing system on carcass yields. Several studies also reported that body weight, body weight gain, and the proportion of breast meat of birds from the free-range system were significantly lower than of those reared in indoor floor system (Grashorn and Serini, 2006; Dou et al., 2009; Wang et al., 2009). Conversely, some other researchers have found that free-range chickens had the higher live weight, breast, and thigh-drumstick weights compared to the conventional system (Alvarado et al., 2005; Santos et al., 2005; Połtowicz and Doktor, 2011; Lin et al., 2014; Comert et al., 2016) and also there are some other results reported by different researchers that were consistent with findings of the current study as they claimed that most of the carcass yield, especially body weight, did not show significant changes (Cheng et al., 2008; Chen et al., 2013; Fu et al., 2015).

The meat quality attributes including color values, pH measures, and water holding capacity were evaluated in this experiment because these parameters can have important effects on the characteristics of fresh chicken meat at the point of sale (Kim et al., 2020).

In the present study, the initial and ultimate pH of breast meat were slightly affected by two rearing systems but the changes were not significant and this finding was consistent with the results of many studies (Cheng et al., 2008; Husak et al., 2008; Ponte et al., 2008; Wang et al., 2009; Mikulski et al., 2011; Li et al., 2017). Although the initial and ultimate pH values were not significantly different between the two rearing systems, their variation obviously showed a different pattern that could be deduced why some studies have concluded that meat from broilers reared in the free-range system had lower shelf-life stability compared with conventionally reared broiler meat (Alvarado et al., 2005; Husak et al., 2008; Funaro et al., 2014). Because pH, microorganism content, and oxidation are intra-dependent factors and pH alteration during storage can be resulted by proteolytic degradation or fat oxidation which is decreases shelf-life of meat (Kim et al., 2020). This notable different pattern in pH change in breast meat of free-range birds might be the result of intensive physical activity and more pre-slaughter stress in comparison to the conventional broilers (da Silva et al., 2017; Kim et al., 2020). Pre-slaughter stress and physical activity in free-range chicken may lead to the range of

chemical changes based on the conversion of ATP to ADP and also anaerobic glycolysis of glycogen storage in muscles decreases pH due to lactic and pyruvic acid production so finally it can change the meat acidity during the rigor mortis.

The influence of the two studied rearing systems on meat quality did not reveal a significant difference in the cooking loss and water holding capacity parameters, although, drip loss parameter was affected by rearing systems significantly. The result of water holding capacity concurs with similar studies in comparing breeding methods (Castellini et al., 2002; Cheng et al., 2008; Dou et al., 2009; Wang et al., 2009; Stadig et al., 2016; Li et al., 2017). Water holding capacity is an important attribute of meat quality, if water holding capacity is poor, meat and meat products will lack juiciness. As slow-growing chickens are better suited to the free-range system (Castellini et al., 2008; Kingori et al., 2010; Bogosavljevi-Boskovic et al., 2012), but Fanatico et al. (2007) claimed that slow-growing birds had weaker water-holding capacity but their meat is more tender than the fast-growing birds.

The absence of significant differences in the carcass yield and other studied traits between two different systems are may be due to short rearing period with outdoor access. An experiment was conducted to evaluate the effect of free-range days on growth performance, carcass yield, and meat quality; and it was reported that increasing free-range length advantageously affects breast yield, but decreases thigh, and foot yields as well as the water-holding capacity of the thigh. No evidence was found that free-range days can change growth performance and meat quality (except water holding capacity) (Tong et al., 2014).

It can be demonstrated that free-range rearing system can modify the color values in chicken meat as the findings showed the breast meat from free-range broilers had higher yellow color and much lower red color than another rearing system (Brown et al., 2008; Połtowicz and Doktor, 2011; Funaro et al., 2014; da Silva et al., 2017). Of course, it should not be ignored that there were some peculiarities in results reported by authors about meat color values in comparing two standard and free-range meat yield.

According to the present result, raw breast meat from free-range birds had significantly lower fat content in comparison to conventional broilers, which could be important to consumers concerned with fat intake. The obtained results in previous studies suggested that the free-range rearing system was more favorable than the conventional system, as it resulted in significantly higher protein content and a lower fat content of chicken meat (Bogosavljevi-Boskovic et al., 2012) however, no significant differences were observed for protein and ash content of the raw breast in the present study.

CONCLUSION

Although the quantitative carcass traits showed less significant differences between the two groups, some main qualitative traits including apparent characteristics such as all color coordinates and drip loss were significantly affected by the free-range system. Moreover, fat percentage of raw breast meat was significantly lower in the free-range production system, therefore, it could be healthier. Accordingly, no significant differences in the amount of meat produced in two rearing systems showed that the conventional production system in the poultry industry can be successfully replaced by alternative systems such as free-range without production loss. Also, the different chemical composition of meat in the free-range system may become more attractive in terms of increasing healthy food demand in the world industry. Ultimately, further studies are suggested to investigate consumer preferences, especially in terms of sensory evaluation of meat from the two production systems.

DECLARATIONS

Competing interests

We certify that there is no conflict of interest with any financial organization regarding the aspects discussed in the manuscript.

Authors' contributions

Alireza Ehsani designed the experiments, guided the research team, and edited the final draft of the manuscript. Peymaneh Davoodi performed field and lab researches, analyzed the data, and wrote the paper. Finally, all authors read and approved the final manuscript.

REFERENCES

Alvarado CZ, Wenger E, and O-Keefe SF (2005). Consumer perceptions of meat quality and shelf-life in commercially raised broilers compared to organic free range broilers. Proceedings of the XVII European Symposium on the Quality of Poultry Meat, Doorwerth, Netherlands, pp. 257-261. Available at: https://www.cabi.org/Uploads/animal-science/worlds-poultry-science-association/WPSA-the-netherlands-2005/25.pdf

Berg C (2001). Health and welfare in organic poultry production. Acta Veterinaria Scandinavica Supplementum, 95: 37-45. DOI: https://www.doi.org /10.1186/1751-0147-43-S1-S37

Bogosavljevi-Boskovic S, Rakonjac S, DoskoviĆ V, and PetroviĆ MD (2012). Broiler rearing systems: A review of major fattening results and meat quality traits. World's Poultry Science Journal, 68(2): 217-228. Available at: https://www.cambridge.org/core/article/broiler-rearing-systems-a-review-of-major-fattening-results-and-meat-quality-traits/9F9B98C9AFA0601C2DFA1EA4EF328962

Bogosavljevic-Boskovic S, Pavlovski ZP, Etrovic MD, Doskovic V, and Rakonjac S (2010). Broiler meat quality: Proteins and lipids of muscle tissue. African Journal of Biotechnology, 9(54): 9177-9182. DOI: https://www.doi.org /10.5897/AJB.9000089

Brown SN, Nute GR, Baker A, Hughes SI, and Warriss PD (2008). Aspects of meat and eating quality of broiler chickens reared under standard, maize-fed, free-range or organic systems. British Poultry Science, 49(2): 118-124. DOI: https://www.doi.org /10.1080/00071660801938833

Castellini C, Berri C, Le Bihan-Duval E, and Martino G (2008). Qualitative attributes and consumer perception of organic and free-range poultry meat. World's Poultry Science Journal, 64(4): 500-512. Available at: https://www.cambridge.org/core/article/qualitative-attributes-and-consumer-perception-of-organic-and-freerange-poultry-meat/C17F5D0B7D747DC2578B1B0672F4DFEE

Castellini C, Mugnai C, and Dal Bosco A (2002). Effect of organic production system on broiler carcass and meat quality. Meat Science, 60(3): 219-225. Available at: http://www.sciencedirect.com/science/article/pii/S0309174001001243

Chen X, Jiang W, Tan HZ, Xu GF, Zhang XB, Wei S, and Wang XQ (2013). Effects of outdoor access on growth performance, carcass composition, and meat characteristics of broiler chickens. Poultry Science, 92(2): 435-443. DOI: http://www.dx.doi.org/10.3382/ps.2012-02360

Cheng FY, Huang CW, Wan TC, Liu YT, Lin LC, and Luo Chyr CY (2008). Effects of free-range farming on carcass and meat qualities of black-feathered Taiwan native chicken. Asian-Australas Journal of Animal Science, 21(8): 1201-1206. DOI: https://www.doi.org/10.5713/ajas.2008.80080

Choo YK, Kwon HJ, Oh ST, Um JS, and Kim BG (2014). Comparison of growth performance, carcass characteristics and meat quality of Korean local chickens and silky fowl. Asian-Australas Journal of Animal Science, 27(3): 398-405. DOI: https://www.doi.org/10.5713/ajas.2013.13638

Comert M, Sayan Y, Kirkpinar F, Bayraktar OH, and Mert S (2016). Comparison of carcass characteristics, meat quality, and blood parameters of slow and fast grown female broiler chickens raised in organic or conventional production system. Asian-Australas Journal of Animal Science, 29(7): 987-997. DOI: https://www.doi.org /10.5713/ajas.15.0812

National Research Council (NRC) (1994). Nutrient requirements of poultry: Ninth revised edition, 1994. Washington, DC: The National Academies Press, DOI: https://www.doi.org/10.17226/2114

da Silva DCF, de Arruda AMV, and Goncalves AA (2017). Quality characteristics of broiler chicken meat from free-range and industrial poultry system for the consumers. Journal of Food Science and Technology, 54(7): 1818-1826. Available at: https://www.link.springer.com/article/10.1007/s13197-017-2612-x

Dou TC, Shi SR, Sun HJ, and Wang KH (2009). Growth rate, carcass traits and meat quality of slow-growing chicken grown according to three raising systems. Animal Science Papers and Reports, 27(4): 361-369. Available at: http://agro.icm.edu.pl/agro/element/bwmeta1.element.agro-6e21f956-2203-451e-86c3-fd03229a6bab

Fanatico AC, Pillai PB, Emmert JL, and Owens CM (2007). Meat quality of slow- and fast-growing chicken genotypes fed low-nutrient or standard diets and raised indoors or with outdoor access. Poultry Science, 86(10): 2245-2255. DOI: http://www.dx.doi.org/10.1093/ps/86.10.2245

Fu D, Zhang D, Xu G, Li K, and Wang Q (2015). Effects of different rearing systems on meat production traits and meat fiber microstructure of Beijing-you chicken. Animal Science Journal, 86(7): 729-735. DOI: https://www.doi.org 10.1111/asj.12347

Funaro A, Cardenia V, Petracci M, Rimini S, and Rodriguez-Estrada MT (2014). Comparison of meat quality characteristics and oxidative stability between conventional and free-range chickens. Poultry Science, 93(6): 1511-1522. DOI: https://www.doi.org /10.1111/asj.12347

Grashorn MA, and Serini C (2006). Quality of chicken meat from conventional and organic production. Conference Proceed. Available at: EPC 2006 - 12th European Poultry Conference, Verona, Italy, 10-14 September, 2006 pp.paper 67 ref.6

Husak RL, Sebranek JG, and Bregendahl K (2008). A survey of commercially available broilers marketed as organic, free-range, and conventional broilers for cooked meat yields, meat composition, and relative value. Poultry Science, 87(11): 2367-2376. DOI: http://www.dx.doi.org/10.3382/ps.2007-00294

Ishamri I, and Seon-Tea T (2017). Poultry meat quality in relation to muscle growth and muscle fiber characteristics. Korean Journal for Food Science of Animal Resources, 37(6): 873-883. Available at: https://www.ncbi.nlm.nih.gov/pubmed/29725209

Kim HJ, Kim HJ, Jeon J, Nam KC, and Shim KS (2020). Comparison of the quality characteristics of chicken breast meat from conventional and animal welfare farms under refrigerated storage. Poultry Science, 99(3): 1788-1796. Available at: http://www.sciencedirect.com/science/article/pii/S0032579119579202

Kingori AM, Wachira AM, and Tuitoek JK (2010). Indigenous chicken production in Kenya: A review. International Journal of Poultry Science, 9(4): 309-316. DOI: https://www.doi.org /10.3923/ijps.2010.309.316

Lee MH (1995). Official methods of analysis of AOAC International (16th edn) Trends in Food Science & Technology. Cunniff, AOAC International, DOI: https://www.doi.org /10.1016/0924-2244(95)90022-5

Li Y, Luo C, Wang J, and Guo F (2017). Effects of different raising systems on growth performance, carcass, and meat quality of medium-growing chickens. Journal of Applied Animal Research, 45(1): 326-330. DOI: https://www.doi.org/10.1080/09712119.2016.1190735.

Lin CG, Kuo HY, and Wan TC (2014). Effect of free-range rearing on meat composition, physical properties and sensory evaluation in Taiwan game hens. Asian-Australasian Journal of Animal Sciences, 27(6): 880-885. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4093180/

Miele M (2011). The taste of happiness: Free-range chicken. Environment and Planning A: Economy and Space, 43(9): 2076-2090. DOI: https://www.doi.org/10.1068/a43257

Mikulski D, Celej J, Jankowski J, Majewska T, and Mikulska M (2011). Growth performance, carcass traits and meat quality of slower-growing and fast-growing chickens raised with and without outdoor access. Asian-Australas Journal of Animal Science, 24(10): 1407-1416. DOI: https://www.doi.org/10.5713/ajas.2011.11038

Połtowicz K, and Doktor J (2011). Effect of free-range raising on performance, carcass attributes and meat quality of broiler chickens. Animal Science Papers and Reports, 29(2): 139-149. Available at: https://journals.indexcopernicus.com/search/article?articleId=72754

Ponte PIP, Rosado CMC, Crespo JP, Crespo DG, and Mourão JL (2008). Pasture intake improves the performance and meat sensory attributes of free-range broilers. Poultry Science, 87(1): 71-79. DOI: http://www.dx.doi.org/10.3382/ps.2007-00147

Saleh EA, Ellakany HF, El-Far AH, Kadry S, and Elbestawy AR (2015). Meat quality and biochemical parameters related to human health under organic broiler production. Global Veterinaria, 14(3): 409-417. DOI: https://www.doi.org/10.5829/idosi.gv.2015.14.03.93115

Santos AL, Sakomura NK, Freitas ER, Fortes CMS, and Carrilho ENVM (2005). Comparison of free range broiler chicken strains raised in confined or semi-confined systems. Revista Brasileira de Ciência Avícola, 7: 85-92. Available at: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-635X2005000200004&nrm=iso

Srednicka-Tober D, Baranski M, Seal C, Sanderson R, and Benbrook C (2016). Composition differences between organic and conventional meat: A systematic literature review and meta-analysis. British Journal of Nutrition, 115(6): 994-1011. DOI: https://www.doi.org/10.1017/S0007114515005073

Stadig LM, Rodenburg TB, Reubens B, Aerts J, and Duquenne B (2016). Effects of free-range access on production parameters and meat quality, composition and taste in slow-growing broiler chickens. Poultry Science, 95(12): 2971-2978. DOI: https://www.doi.org /10.3382/ps/pew226

Tong HB, Wang Q, Lu J, Zou JM, and Chang LL (2014). Effect of free-range days on a local chicken breed: Growth performance, carcass yield, meat quality, and lymphoid organ index. Poultry Science, 93(8): 1883-1889. DOI: https://www.doi.org /10.3382/ps.2013-03470

Wang KH, Shi SR, Dou TC, and Sun HJ (2009). Effect of a free-range raising system on growth performance, carcass yield, and meat quality of slow-growing chicken. Poultry Science, 88(10): 2219-2223. DOI: https://www.doi.org/10.3382/ps.2008-00423

Wierbicki E, and Deatherage FE (1958). Water content of meats, determination of water-holding capacity of fresh meats. Journal of Agricultural and Food Chemistry, 6(5): 387-392. DOI: https://www.doi.org/10.1021/jf60087a011

Xing T, Gao F, Tume RK, Zhou G, and Xu X (2019). Stress effects on meat quality: A mechanistic perspective. Comprehensive Reviews in Food Science and Food Safety, 18(2): 380-401. Available at: https://onlinelibrary.wiley.com/doi/abs/10.1111/1541-4337.12417

Yeung RMW, and Morris J (2001). Consumer perception of food risk in chicken meat. Nutrition and Food Science, 31(6): 270-279. DOI: https://www.doi.org/10.1108/00346650110409092

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 631-642, December 25, 2020

Research Paper, PII: S2322455X2000072-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.72

Effect of *Amphora coffeaeformis* and *Star anise* as Dietary Supplementson the Immunity and Growth Performance of Broiler Chickens

Sherif Mohamed Shawky[1*], Said Ibrahim Fathalla[1], Sahar Hassan Orabi[2], Huda Hassan El-Mosalhi[1], and Ibrahim Said Abu-Alya[1]

[1]*Department of Physiology, Faculty of Veterinary Medicine, University of Sadat City, Egypt.*
[2]*Department of Biochemistry, Faculty of Veterinary Medicine, University of Sadat City, Egypt.*

[*]Corresponding author's Email: shsh00076@yahoo.com ; ORCID:0000-0003-0629-7063

Received: 07 Nov. 2020
Accepted: 20 Dec. 2020

ABSTRACT

The present study was designed to evaluate the impacts of daily dietssupplemented with *Amphora coffeaformis* and *Star anise* on growth performance and immunity of Cobb broiler chickens. *Amphora coffeaformis* is considered a potent free radical scavenger due to the presence of β-carotene and fucoxanthin, which are used widely as food additives. *Star Anise* has a natural antioxidant, which can also be used for the chemo-prevention of disease occurring due to oxidative deterioration. A total of 270 broiler chickens were divided into three groups, each with three replicates of 30 birds. The control group (G1) was given the basal diet, the *Amphora* group (G2) received *Amphora* in a dose of 1g/Kg in the ration, and the *Star anise* group (G3) received *Star anise* in a dose of 2g/Kg in the ration. The results indicated that *Amphora* and *Star anise* significantly improved the final body weight, weight gain, and feed conversion ratio, total white blood cells count, phagocytic activity percentage, phagocytosis index in plasma, IgM, IgG, and A/G ratio in serum. In addition, *Amphora* and *Star anise* significantly increased mRNA expression of hepatic growth hormone gene, insulin-like growth factor-1 (IGF-1) genes (IGF1), and mRNA expression of splenic interferon-gamma (INF-γ) and Interleukin 12 (IL-12p35) genes from broiler chickens, compared to the control group. In conclusion, the use of fed additives, such as *Amphora coffeaeformis*and *Star anise* in the diet of broiler chickens for 35 days was sufficient to improve broiler growth performance and could modulate their immunity.

Keywords: *Amorpha coffeaeformis*, Broiler chickens, Diet supplementation, Growth performance, Immunity, *Star anise*

INTRODUCTION

Global food production is facing a greater challenge than ever before. Protein in particular is one of the most important nutrients in animal and human life (FAO, 2018).

Broiler production as a source of protein has become an important issue in many growing countries. However, the recent increase in the costs of traditional feed was once a major contributor to net returns from the poultry business. Feed accounted for 70-80 percent of the total costs of poultry production (Bolu and Balogun, 2004). It was found that reducing feed costs using cheaper and more unconventional feed was once a required problem for commercial poultry production (Bhatta and Sharma, 2001). The feed additives are a collection of nutrients and non-nutrients compounds that help to increase the competence of feed usage and thus reduce the high feed costs. In recent years, these additives have played an important role as feed supplements for many purposes in poultry production (Zhang et al., 2009).

Microalgae were identified as microscopic, unicellular, and photosynthetic organisms and can grow in saline and freshwater which provide a rich supply of nutrients and biologically active compounds, such as proteins, amino acids, polyunsaturated fatty acids, microelements, vitamins, antioxidants called carotenoids, which have long records of human application as a food (Belotti et al., 2014). Microalgae of exclusive species could be properly involved in poultry diets, which can affect advisably on birds' health, performance, and comfort of chickens' meat and egg (Abdelnour et al., 2019).

Microalgae were considered to be an adequate supply of a large number of metabolites that were suitable for animal feed. These metabolites included proteins,

carbohydrates, fats, vitamins, and minerals (Andrade et al., 2018).

Currently, microalgae are receiving more attention in the market as nutraceuticals and fitness meals. Several microalgae, such as Spirulina, Chlorella, and *Amphora* are grown commercially for the production of algal products such as β-carotene, lutein, and phycocyanin (Hirata et al., 2000). *Amphora* is a major genus of diatoms of marine and freshwater origin (Parnell and Trevor, 2007).

*Amphora coffeaformi*s is one of the most common alkaline fresh ecosystems and brackish-water localities (Bhosleac et al., 1993). *Amphoracoffeaformis* has strong antioxidant activity against lipid peroxidation (Sugiharto et al., 2018), and is rich in polyunsaturated fatty acids (PUFAs), especially Docosahexaenoic (DHA), Eicosapentaenoic (EPA), and α-linolenic acid (Lee et al., 2009).

The microalga was a source of bioactive compounds including *Amphora coffeaeformis*, especially the carotenoids, sulfated polysaccharides, polyunsaturated fatty acids, α-tocopherol, especially β-glucans, in addition to vitamins C and E (El-Sayed et al., 2018)

Several studies on diatoms such as Amphora showed the possibility of their extract to use in both protective and antioxidant agents (Mekkawy et al., 2020), except for their antibacterial (Ayoub et al., 2019), antiviral (Abdel-Wahab, 2018), anti-inflammatory factors (Lauritano et al., 2016) and their dietary supplements (Selvaraj et al., 2013).

Amphora coffeaeformis was considered as a potent radical scavenger due to the presence of β-carotene and fucoxanthin, which are widely used as food additives in addition to the various nutraceutical applications such as pro-vitamin (Jaswir et al., 2011). The oral administration of *Amphora coffeaeformis* at three attentions (10, 20, and 30g / kg diet) in Nile tilapia (*O. niloticus*) diets led to enhance in growth performance, feed efficiency, and serum lysozyme (Ayoub et al., 2019).

Herbs are mixed into poultry diets to replace synthetic products and to stimulate or promote the efficient use of feed nutrients, which may subsequently result in faster body weight gain, higher production rates, and increased feed efficiency. In addition, active ingredients in herbs could improve digestion and stimulate the immune functions of broilers (Ghazalah and Ali, 2008; Shawky et al., 2020 a, b).

Star anise is a medium-sized evergreen tree that is native to southwest China and is also extensively cultivated in the subtropical and tropical regions of Asia (Benmalek et al., 2013; Elmasry et al., 2018). It has been shown that *star anise* promoted digestion, and has antioxidant, antibacterial, antiparasitic, antipyretic, and antifungal properties (Ertas et al., 2005; Mohammed, 2008).

However, anethole is an important compound in the *Star anise*, the other important compounds in the seeds include p-anisaldehyde, anise alcohol, acetophenone, pinene, and limonene. Seeds have excellent supplies of minerals such as calcium, iron, manganese, magnesium, zinc, potassium, and copper. This essential spice contains precise amounts of antioxidant nutritional vitamins such as vitamin C and vitamin A (Zhou et al., 2005). Shikimic acid, which is a component in *Star anise*, is one of the best components of the antiviral drug Tamiflu for combating avian influenza (Ohira et al., 2009; Borah, 2015). *Star anise is* pronounced to possess antioxidant residences (Chempakam and Balaji, 2008) as well as a tremendous anticancer (Shu et al., 2010).

MATERIALS AND METHODS

Ethical approval

The experimental design was approved by the Ethical Research Committee of the Faculty of Veterinary Medicine, the University of Sadat City, Egypt with approval number VUSC-014-2-18.

Experimental animals

The current study was conducted in the Department of Physiology, the University of Sadat City, Faculty of Veterinary Medicine, Sadat City, Egypt, on 270 Cobb broiler chickens that were one-day-old and had an average weight of 45.0 ±2.0 gram (Cobb strain) of the Misr-El-Arabia Company for Poultry.

Feed and water were supplied *ad libitum*, and synthetic lights were supplied 24 hours a day for the first 15 days to assist newly hatched chickens to commence drinking and eating. After two weeks, one hour of darkness was applied once a day. The chickens were reared at 33°C to 34°C for the first week, and then regularly decreased by 2-3°C per week until the temperature reached around 21 to 23°C, while the relative humidity was maintained around 55 to 65 percent. Chickens were reared in the poultry house, equipped with feeders, drinkers, and wood shaves used as bedding material. Strict sanitation practices were applied during some stages of the experiment. The chickens were reared under comparable environmental conditions. The experimental house was warm, dry, and free from drafts. Sparkling air provided some sort of ventilation to remove excess moisture and ammonia from the facility.

Nutrition

Chickens were feed starter rations that contained all of the necessary nutrients needed for maintenance, growth, and reproduction from day one to day 14. At the beginning of the third-week, chickens fed on the finisher ration (Table 1).

Table 1. The ingredients and chemical analysis of basal diet of broiler chickens

Ingredients and composition (%)	Starter	Finisher
Corn	55.59	61.07
Soybean meal	37.32	31.83
Soy oil	2.98	3.41
Lime stone	1.21	1.42
Dicalcium Phosphate	1.60	1.16
DL. Methionine	0.20	0.10
*Vitamin and Minerals	0.60	0.60
Sodium chloride	0.23	0.18
Sodium bicarbonate	0.27	0.23
Chemical Analysis (%)		
Metabolizable energy(ME) kcal/kg	2950	3050
Crude Protein (%)	21.20	19.16
Lysine (%)	1.14	1.01
Methionine (%)	0.50	0.39
Methionine and Cysteine (%)	1.03	0.84
Available Methionine + Cysteine (%)	0.85	0.71
Calcium (%)	0.93	0.90
Available Phosphate (%)	0.44	0.35

*Supplied per kilogram of diet: vitamin A, 1,500 IU; cholecalciferol, 200 IU; vitamin E, 10 IU; riboflavin, 3.5 mg; pantothenic acid10 mg; niacin, 30 mg; cobalamin, 10 µg; choline chloride, 1,000 mg; biotin, 0.15 mg; folic acid, 0.5 mg; thiamine 1.5 mg; pyridoxine 3.0 mg; iron, 80 mg; zinc, 40 mg; manganese, 60 mg; iodine, 0.18 mg; copper, 8 mg; selenium, 0.15 mg.

Preparation of *Amphora coffeaeformis* extract

Amphoracoffeaeformis extract was obtained from the Algae Production Unit (APU), National Research Institute (center), Cairo, Egypt. The extract was previously identified for its phytochemical constitute using liquid chromatography-mass spectrometry (Mekkawy et al., 2020).

Preparation of *Star anise*

The dried *Star anise* was purchased from a local market and was added to the diet after grinding into powder.

Animal grouping

From the seventh day of the experiment, the chickens were divided into three groups with three replicates of 30 birds each. The chickens were raised on the floor with given water and diet *ad libitum*. All chickens were vaccinated against Newcastle Disease (ND) and Infectious Bronchitis (IB) strains HIB120 at ninth days of age and against infectious bursal disease (Gumboro) at the 13thday of age. From day 16 to 35, no vaccination was given against any disease. Chickens were randomly divided into three experimental groups, including group 1 (control group), which chickens only received basic ration; group 2, which ration was mixed with *Amphora* at a dose of 1g / kg feed (Mekkawy et al., 2020), and group 3, in which the chickens received a ration mixed with *Star anise* at a dose of 2 g /kg feed (Alhajj et al., 2015; Ding et al., 2017).

Growth performance parameters

The growth performance parameters of broiler chickens have consisted of Body Weight (BW, gram), Body Weight Gain (BWG), Feed Intake (FI), and Feed Conversion Ratio (FCR). All chickens in groups had been weighed individually at the start and the end of the experiment. The body weight gain of chickens (expressed in grams) in each group was calculated by the difference between 2 successive weights (Nwanna, 2003).

Weight gain = (W2- W1)

Where W1 is the mean chicken weight at the beginning of the experiment and W2 is the mean chicken weight at the end of the experiment**.**

The experimental diets were offered regularly to each group. The feeds offered were measured daily, and at the end of the week, the weekly feed consumption was determined by the difference between the weight of feed offered and remained part. The FCR was calculated by dividing the amount of FI (g) during the entire experimental period by the total BWG (g) as outlined by Abd El-Wahed (1998).

FCR = total FI (g) of chicken / total BWG (g) of chicken.

Sample collection

On day 35, blood samples were taken from the wing vein of 10 chickens per group with anticoagulant (EDTA) to obtain plasma. The plasma was separated from the blood cells by centrifugation at 3.000 rounds per minute (rpm) for 30 minutes to determine haematological and biochemical parameters. Thereafter, chickens were slaughtered, and then the liver, and spleen were removed

to estimate the mRNA expression of GH, IGF-1, IFN-γ, IL - 12p35 according to Cinthia et al. (2013).

Determination of haematological parameters

Red Blood Cells (RBCs), White Blood Cells (WBCs), Haemoglobin (Hb) concentration, and Packed Cell Volume (PCV) were estimated using automated technical analyserDirui Bcc-3600. The Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), and Mean Corpuscular Haemoglobin Concentration (MCHC) were determined according to the method describing by Feldman et al. (2000). Differential leukocyte counts (heterophils, eosinophils, basophils, lymphocytes, and monocytes) were counted on blood smears stained with May-Grunwald-Giemsa (Tavares-Dias and Moraes, 2003). Phagocytosis of polymorph nuclear cells using *Candida Albicans* was performed in accordance with the method described by Sornplang et al. (2015).

Phagocytic Activity % = (Number of Heterophils ingesting candida ×100)/ Total number of Heterophils.

Phagocytic index = The total number of ingested candida / Number of active Heterophils.

Determination of biochemical parameters

The assay of total plasma proteins (g/dl) and albumin (g/dl) was carried out by a colorimetric method using commercial Diamond diagnostics kits according to the method described by Cannon et al. (1974) and Doumas et al. (1971), respectively.Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) enzymes were determined colorimetrically using commercial kits of Bio diagnostics according to Reitman and Frankel, (1957). Creatinine (mg/dl) was determined by the colorimetric method using commercial kits of Biomed diagnostics according to Young (2001).Urea (mg/dl) was measured by the colorimetric method using commercial kits of Diamond diagnostics according to the method of Chaney et al. (1962). Protein electrophoresis profile was carried out by a Polyacrylamide Gel Electrophoresis according to Lewis et al. (2006).

Gene expression

Analysis of the mRNA expression of genes of Hepatic Growth Hormone and Insulin-like Growth Factor-1

The analysis of the mRNA of the expression of Growth Hormone (GH) and Insulin Growth Factor-1 (IGF-1) was performed at the Central Laboratory of the Faculty of Veterinary Medicine, Benha University, in Egypt. Liver samples were dissected from each group and then immediately frozen at -80°C. Total RNA was extracted from the frozen liver using the RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol. The quantity and quality of RNA were determined using SPECTROstar Nanodrop. Single-stranded cDNA was synthesized from 1000 ng of total RNA according to the manufacturer's protocol for High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cycling conditions were 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C. Total RNA and cDNA samples were then stored at -80° C until used. Expression of hepatic GH and IGF-1 genes was analyzed by real time-PCR using sense and antisense primers as previously described (Gasparino et al., 2014) by using the primers sets including GH sense (5 -′AAGGGATCCAAGCTCCTGAT-3′), and antisense (5 -′ATAACCACGTCCCTCAGTGC-3′); IGF-1 sense (5 -′ CACCTAAATCTGCACGCT-3′), and antisense (5 -′ CTTGTGGATGGCATGATCT-3′); and βactin as a housekeeping gene, sense (5ACCCCAAAGCCAACAGA-3′) and anti-sense (5 -′ CCAGAGTCCATCACAATACC-3′). PCR reactions for each gene were performed for each sample analyzed. Each PCR reaction consisted of 1.5 µ l of 1µg/µ l cDNA, 10 µ l SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen), and one µMof each forward and reverse primer for GH and IGF-1 genes while one µM forward and 1.5 µM reverse primer for βactingene and nuclease-free water to a final volume of 20 µl. The reactions were then analyzed on an Applied Biosystems 7500 Fast Real-time PCR Detection system under the conditions of 95°C for ten minutes (holding stage) and 40 cycles of 95°C for 15 seconds (denaturation stage) followed by 60°C for one minute (annealing and extension stage). Changes in gene expression were calculated from the obtained Cycle threshold (Ct) values, which were provided by real-time PCR instrumentation using the comparative Threshold cycle (TC) method for a reference (housekeeping) gene (βactin) (Gasparino et al., 2014).

Analysis of mRNA expression of splenic interferon-gamma and Interleukin 12 genes

Spleen samples were dissected from all groups and then immediately frozen at -80°C. Total RNA was extracted from the frozen spleen using the RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol. The quantity and quality of the RNA were determined by using Spectrostar nanodrop Single-stranded cDNA was synthesized from 1000 ng of total RNA according to the manufacturer's protocol for High Capacity cDNA Reverse

Transcription Kits (Applied Biosystems). Cycling conditions were 25°C for 10 minutes, 37°C for 120 minutes, and 85° C for 5 minutes. Afterward, total RNA and cDNA samples were stored at -80° C until use. Each PCR reaction consisted of 1.5 µl of one µg/µl cDNA, tenµl SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen), one µM of each forward and reverse primer for INF-γ and IL-12p35 genes while one µM of forward and 1.5 µM reverse primer for βactin gene and nuclease-free water to a final volume of 20 µl. The Reactions were then analyzed on the Applied Biosystem 7500 Fast Real-time PCR Detection system under the conditions of 95°C for ten minutes (holding stage) and 40 cycles of 95°C for 15 seconds (denaturation stage) followed by 60°C for one minute (annealing and extension stage). Changes in gene expression were calculated from the obtained Cycle threshold (Ct) values provided by real-time PCR instrumentation using the comparative CT method for a reference (housekeeping) gene (βactin) (Gasparino et al., 2014).

Statistical analysis

The results were expressed as Mean ± Standard Error (SE). The relations between means in different groups were tested using a one-way analysis of variance (ANOVA). Duncan test was used for finding the significant differences in SPSS, 16 version. The P-values at 0.05 or lesser were considered significant.

RESULTS

The data in table 2 presented that the *Amphora* and *Star anise* supplementation resulted in a significant increase ($p < 0.05$) in the final BW, BWG, and also a significant decrease in the FCR compared to the control group ($p < 0.05$). The data in table 3 indicated that the Amphora and Star anise supplement did not significantly affect the erythrogram. The data in table 4 revealed that the *Amphora* and *Star anise* supplementation resulted in a significant increase ($p <0.05$) in total WBCs count, percentage of heterophils, H/L ratio, globulins (alpha 1 globulin, alpha 2 globulin, beta globulin, and gamma globulin), IgG, IgM, phagocytic activity, phagocytic index, while the total protein and albumin, lymphocyte percentage, and platelets count were not significantly affected. The data in table 5 revealed that the *Amphora* and *Star anise* supplementation did not significantly affect urea, creatinine, AST, and ALT ($p > 0.05$). The data in table 6 revealed that the *Amphora* and *Star anise* supplementation significantly increasedthe expression of hepatic GH and IGF1, and increased splenic INF-Gamma and interleukin12p35 compared to the control group ($p>0.05$).

Table 2. Effect of *Amphora* and *Star anise* supplementation on growth performances of broiler

Groups	Control group	*Amphora* group	*Star anise* group
Initial body weight (g)	192.83 ± 1.37a	195.08 ± 1.23a	194.01 ± 1.30a
Final body weight (g)	2054.9 ± 6.74c	2529.3 ± 10.20a	2297.8 ± 10.59b
Weight gain (g)	1824.60 ± 11.79^{c}	2349.90 ± 12.67^{a}	2195.90 ± 17.55^{b}
FCR	2.43 ± 0.03^{a}	1.62 ± 0.01^{c}	1.82 ± 0.02^{b}

In the same row, Mean ± Standard Error with different letters superscripts are significantly different at $P < 0.05$. FCR: Feed Conversion Rate.

Table 3. Effect of *Amphora* and *Star anise* supplementation on erythrogram of broiler

Groups	Control group	*Amphora* group	*Star anise* group
RBCs count ($10^6/mm^3$)	2.45 ± 0.08^{a}	2.60 ± 0.06^{a}	2.59 ± 0.07^{a}
PCV %	29.06 ± 0.61^{b}	30.94 ± 0.49^{a}	29.59 ± 0.62^{ab}
Hb (g/dl)	11.29 ± 0.20^{a}	11.68 ± 0.21^{a}	11.61 ± 0.17^{a}
MCV (μ^3)	111.50 ± 1.94^{a}	116.29 ± 1.62^{a}	116.40 ± 1.82^{a}
MCH (Pg)	38.94 ± 0.62^{a}	38.04 ± 0.85^{a}	37.86 ± 0.70^{a}
MCHC %	30.73 ± 0.27^{a}	31.45 ± 0.30^{a}	30.64 ± 0.39^{a}

In the same row, Mean±Standard Error with different letters superscripts are significantly different at $p<0.05$. RBCs: Red Blood Cells; PCV: Packed Cell Volume; MCV: Mean Corpuscular Volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

Table 4. Effect of *Amphora* and *Star anise* supplementation on immunity profile of broiler chicken

Groups	Control group	*Amphora* group	*Star anise* group
WBCs count (10^3/mm^3)	20.55 ± 0.17^c	32.99 ± 0.41^a	29.03 ± 0.42^b
Lymphocytes (%)	68.65 ± 0.74^a	67.80 ± 0.31^a	67.63 ± 0.32^a
Heterophils (%)	17.62 ± 0.34^c	27.01 ± 0.19^a	23.73 ± 0.32^b
H/L ratio	0.40 ± 0.003^a	0.35 ± 0.006^b	0.30 ± 0.007^c
Platelets count (10^3/mm^3)	310.25 ± 11.51^a	298.25 ± 9.20^a	310.85 ± 8.01^a
Total plasma protein (g/dl)	3.32 ± 0.03^a	3.47 ± 0.07^a	3.41 ± 0.06^a
Albumin (g/dl)	1.83 ± 0.03^a	1.67 ± 0.06^a	1.72 ± 0.06^a
Globulin (g/dl)	1.50 ± 0.02^c	1.80 ± 0.02^a	1.68 ± 0.02^b
Alpha 1 globulin (g/dl)	0.24 ± 0.003^c	0.34 ± 0.006^a	0.32 ± 0.008^b
Alpha 2 globulin (g/dl)	0.32 ± 0.004^a	0.32 ± 0.004^a	0.33 ± 0.005^a
Beta globulin (g/dl)	0.42 ± 0.004^c	0.48 ± 0.010^a	0.45 ± 0.004^b
Gamma globulin (g/dl)	0.51 ± 0.016^c	0.66 ± 0.009^a	0.60 ± 0.018^b
IgM (mg/dl)	50.02 ±.0.8^c	90.02 ± 0.5^a	80.47 ± 1.4^b
IgG(mg/dl)	20.95 ± 0.7^c	40.37 ± 0.5^a	40.14 ± 0.4^b
A/G ratio	1.22 ± 0.03^a	0.93 ±0.04^b	1.03 ± 0.04^b
Phagocytic activity (%)	49.79 ± 0.72^c	67.73 ± 0.73^a	63.69 ± 0.46^b
Phagocytic index	1.93 ± 0.050^c	2.83 ± 0.040^a	2.67 ± 0.026^b

In the same row, Mean ±Standard Error with different letters superscripts are significantly different at $p < 0.05$.WBCs: White Blood Cells; IgM: Immunoglobuline M; IgG: Immunoglobuline G; A/G ratio: albumin / globulin ratio; H/L ratio: Heterophils/ Lymphocytes ratio.

Table 5. Effect of *Amphora* and *Star anise* supplementation on liver function tests and kidney function tests of broiler

Groups	Control group	*Amphora* group	*Star anise* group
ALT (U/L)	191.60 ± 4.35^a	183.30 ± 3.67^a	187.60 ± 2.97^a
AST(U/L)	36.60 ± 1.21^a	33.90 ± 1.10^a	33.30 ± 1.15^a
Urea (mg/dl)	9.29 ± 0.10^a	8.91 ± 0.13^a	9.35 ± 0.13^a
Creatinine(mg/dl)	0.45 ± 0.010^a	0.42 ± 0.005^a	0.44 ± 0.006^a

In the same row, Mean ± Standard Error with different letters superscripts are significantly different at $p < 0.05$.ALT: Serum alanine aminotransferase; AST: serum aspartate aminotransferase.

Table 6. Effect of *Amphora* and *Star anise*supplementation on mRNA expression of growth hormone, Insulin-like growth factor1, Interferon gamma, Interleukin12p35 of broiler chicken, data represented as fold change among different experimental groups

Groups	Control group	*Amphora* group	*Star anise* group
GH	1.27 ± 0.11^b	9.11 ± 0.13^a	8.25 ± 0.21^a
IGF1	1.05 ± 0.03^b	3.78 ± 0.08^a	2.73 ± 0.08^a
INF gamma	1.25 ± 0.03^b	7.60 ± 0.15^a	6.36 ± 0.06^a
IL - 12p35	0.90 ± 0.04^b	2.14 ± 0.06^a	1.81 ± 0.05^a

In the same row, mean ± Standard Error with different letters superscripts are significantly different at $P < 0.05$. GH: hepatic growth hormone; IGF-1: insulin-like growth factor-1; INF-γ: splenic interferon-gamma; IL-12p35: Interleukin12p35.

DISCUSSION

Poultry meat is the second largest food product in the world (Manning et al., 2007). In order to endure poultry production the meet global demand, antibiotic replacements were required (Mcdevitt et al., 2006). In the present study, chickens that were fed a diet supplemented with *Amphora coffeaeformis* and *Star Anise* significantly enhanced their growth performance compared to the control group. This result is attributed to the fact that *Amphora coffeaeformis* significantly improved appetite, which led to higher FI and improved growth (Ayoub et al.,

2019). These results were compatible with those reported by Zhao et al. (2004), who concluded that *Amphora*-treated chickens indicated an increased average daily gain and an improvement in the FCR due to the improvement in the digestibility of nutrients.

The improvement in the FCR could also be due to the fact that *Amphora coffeaeformis* contained several nutrients, especially vitamins and minerals that could aid in promoting growth (Belay et al., 1996). The positive effects of *Amphora* could be due to its bioactive compounds, which have antioxidant, anti-inflammatory, antiviral, and antibacterial effects (Rajput and Mishra, 2012; Salahuddin et al., 2017).

Star anise had a positive effect on the live BW, BWG, and FCR of broiler chickens (Al-hajj et al., 2015). The improvement in broiler performance could be due to the active component in *Star anise*, anethole, which could lead to better digestion by inducing the secretion of endogenous enzymes, better absorption, and improved microbial balance in the gut. Amad et al. (2011) stated that the inclusion of essential oils from thyme and *Star anise* resulted in an improvement of the digestibility of the crude protein, crude ash, crude fat, calcium, and phosphorus. This enhancement in the digestibility also led to an increased surface area for absorption in the intestine and improved nutrient absorption. Additionally, it was found that essential oils had a stimulatory effect on pancreatic enzymes (Rao et al., 2003).

The present study revealed no significant difference in blood parameters between the treatment groups the control group. The present results were in agreement with Soltan et al. (2008) and Mekkawy et al. (2020), who reported that there were no significant differences in blood profiles in groups that supplemented with *Star anise* seed and *Amphora*.

In the present study, the addition of *Amphora* and *Star anise* improved the immunity indices in broilers such as phagocytic activity, gamma globulins, IgM, and IgG. *Amphora coffeaeformis*activated the immune system of the chickens and became resistant to pathogenic bacteria, which was consistent with the finding of Mariey et al. (2012) and Jamil et al. (2015). This activation of the immune system could be due to *Amphora*, which was rich in different pigments and polyphenolic compounds, catechin, gallic acid, and p-coumaric acid (El-Sayed et al., 2018). Also, Jaswir et al. (2011) revealed that *Amphora coffeaeformis* was known to be a potent radical scavenger due to the presence of β-carotene and fucoxanthin, which were often used as food additives in addition to the various nutraceutical applications such as pro-vitamin A, antioxidant, anticancer, and anti-obesity.

Feeding chickens with *Star anise* resulted in a significant increase in the immunity indices of all groups. It might be due to the fact that *Star anise* modulates the immune system by stimulating various immune cells such as macrophages, monocytes, Natural Killer Cell (NKC) and effects on cytokines/chemokines in various *in vitro* and *in vivo* trials (Shahrajabian et al., 2019; Sung et al., 2012 a, b).

The results of *Amohora* which activated the immune system agreed with Kang et al. (2013), who found a significant increase in the lymphocyte counts in algae-fed broilers compared to other chickens. The phagocytic activity was also significantly increased in treated animals that were fed with algae and improved the immune response (An et al., 2010). Also, Khan et al. (2012) reported that the immunomodulatory effects of natural feed additives were linked to their ability to increase phagocytosis of potential macrophages, the production of interleukins, interferon-Y, and tumor necrosis factor, increase the secretory metabolism of macrophages, antigen-presenting cells, and antioxidant functions.

The present findings were in accordance with Abdelnour et al. (2019), who noted that IgG and IgM levels were increased in broilers that consumed food containing algae. Immunoglobulins increasing could be because several types of unicellular microalgae are excellent sources of immunoregulatory polysaccharides, such as β-glucan, βcarotene, and vitamin B12, which play a vital role in inflammatory and immune responses in animals and humans, which promote the macrophages activity and immune cells to increase the production of interferon- γ protein. Therefore, *Amphora* could stimulate the ability of the immune system to fight against pathogens and foreign proteins (Mason, 2001), which were in accordance with (Abdo and Zeinb, 2004), who was indicated that the herbal supplements could improve the immune response since globulin levels were used as an indicator of immune responses and a source of antibody production. Soltan et al. (2008) reported that *Star anise* supplementation in the broiler diet significantly increased lymphocyte counts compared to the control group, possibly be due to an aromatic plant like *Star anise* seeds, which contain many essential B-complex nutritional vitamins such as pyridoxine, niacin, riboflavin, thiamine, and magnesium, copper, potassium, manganese, zinc, and iron. In addition, *Star anise* seeds contained the amounts of antioxidant nutritional vitamins, such as vitamin C and vitamin A (Zhou et al., 2016). Herbal Supplements could

increase the immune response as globulin levels have been used as an indicator of immune responses and antibody sources (Abdo and Zeinb, 2004).

The immunity results were consistent with those of Rahmani and Speer (2005), who found a higher percentage of gamma globulins in broilers given herbal ingredients than the control ones. The present results revealed that there was no significant difference between ALT, AST, Urea, and Creatinine in the treated groups compared to the control group, which indicated that *Amphora* and *Star anise* appeared safe and did not have an adverse effect on physiological and nutritional status. Therefore, the utilization of algae in pharmaceutical applications has attracted world wild attention in recent years (Enwereuzoh and Onyeagoro, 2014). *Star anise* will contribute to the development of more phytotherapeutic products that are cheaper, safer, and affordable, and have a lower risk of resistance than conventional therapeutic drugs (Ritter et al., 2014; Sri et al., 2015).

In the current study, the mRNA expression of GH and IGF-1 was significantly increased in the treated chickens compared to the control chickens ($p < 0.05$). Growth hormones and IGF-1 are required to support normal growth (Scanes, 2009). Furthermore, animal growth is closely related to the mRNA expression levels, as intestinal nutrient transporters are required to circulate digestive products from digestion (McCracken and Edinger, 2013).

The results of the current study are compatible with those of Guobin et al. (2011), who reported that IGFs were important positive modulators of body and muscle growth in mammals and chickens.

In the current study, the splenic mRNA expression of IFN-γ was significantly increased in the treated chickens in comparison with the control chickens and the housekeeping gene. Interferon-γ regulated acquired immunity by stimulating lymphocytes and increasing the expression of class II antigens of Major Histocompatibility (MHC). In addition, IFN-γ is a common marker of cellular immunity, and high levels have been correlated with protective immune responses to parasitic infections (Lee et al., 2008). Interferon-gamma (INF) influences the immune system and inhibits tumor growth, and are involved in various immune interactions as inducers, regulators, and effectors of both innate and acquired immunity during the infections (Priyanka and Muralidharan, 2014).

The molecular results of the present study indicated an increase of INF gene transcription levels in the spleen of the treated chickens compared to the control chickens. Blinkova et al. (2001) reported that algae are important positive stimulators for the production of antibodies, cytokines, and also T and B cell activation. Moreover, the available molecular results from INF agreed with Hirahashi et al. (2002), who reported that algae enhanced Natural killer cell functions by INF- γ production and cytolysis. On the other hand, Mohammed AL (2014) stated a significant increase in the INF-γ concentration in the blood serum of both groups of mice treated with two concentrations of herbal additives (1 and 5 mg/kg BW) compared to the control group. Also, Kim et al. (2010) and Lillehoj et al. (2011) reported that supplementing one-day-old chickens diets with medicinal plants indicated higher rates of interleukin interferon (IFN-γ), encoding gene transcripts in comparison with the chickens given a standard diet.

In the current study, the mRNA expression of the IL-12 levels was significantly increased in the treated chickens compared to the control chickens. These results were in agreement with the results of Philbin et al. (2005), who reported that IL-12 differentiates T cells (Trinchieri, 1994), which is known as a factor that activates T cells, that could promote the T cells growth and function. The activation of T cells enhances IFN-γ development (Lesley et al., 2000). The present finding agreed with Ferdous et al. (2008) and Kirshenbaum et al. (2008), who found that adding the herbal additive to broilers' feed increased the expression of IL–12.

CONCLUSION

The supplementation of *Amphora coffeaeformis* and *Star anise* to the diet of broiler chickens for 35 days was sufficient to improve broiler performance by improving BW, BWG, and FCR and to modulate their immunity through increasing total WBC count, the phagocytic activity percentage, the phagocytosis index in plasma, IgM, and IgG, in serum. In addition,*Amphora* and *Star anise* significantly increased the mRNA expression of the hepatic growth hormone gene, the insulin-like growth factor-1 (IGF-1) genes (IGF1), and the mRNA expression of splenic interferon-gamma (INF-γ), and Interleukin12 (IL-12p35) genes.

DECLARATION

Acknowledgments

We appreciate the Faculty of Veterinary Medicine of the University of Sadat City in Egypt.

Competing interests

The authors declared that they have no conflicts of interest.

Author's contribution

Sherif Mohamed Shawky, Said Ibrahim Fathalla, and Sahar Hassan Orabi have participated in the creation of the conception, design, and writing of the manuscript. Sherif Mohamed Shawky, Said Ibrahim Fathalla, Sahar Hassan Orabi, Huda Hassan El-Mosalhi, and Ibrahim Said Abu-Alya interpreted the data and reviewed the manuscript, while Huda Hassan El-Mosalhi and Ibrahim Said Abu-Alya managed and analyzed the data. Huda Hassan El-Mosalhi, Said Ibrahim Fathalla, and Ibrahim Said Abu-Alya carried out the sampling and management of the samples.

REFERENCES

Abd El-Wahed HM (1998). The use of sorghum or rice bran in broiler and layer diets with reference to enzyme supplementation. Ph. D. Thesis, Faculty of Agriculture, Fayoum, Cairo University, Egypt.Available at:www.fayoum.edu.eg/English/pgsr/AgriAbstractP73.aspx?AspxAutoDetectCookieSupport=1

Abdelnour SA, Abd El-hack ME, Arif M, Khafaga AF, and Taha AE (2019). The application of the microalgae Chlorella spp. as a supplement in broiler feed World's Poultry Science Association. World's Poultry Science Journal, 75:305-318. DOI: https://www.doi.org/10.1017/S0043933919000047

Abdel-Wahab AM (2018). In-vitro studies on antiviral effects of *Galaxauraelongata* marine algae on white spot syndrome virus, Benha Veterinary Medical Journal, 34(1): 162-171. Available at: https://bvmj.journals.ekb.eg/article_54231.html

Abdo M, and Zeinb A (2004). Efficacy of acetic acid in improving the utilization of low protein-low energy broiler diets. Egyptian Poultry Science, 24: 123-141.Available at: https://eurekamag.com/research/004/139/004139795.php

Al-hajj MS, Alhobaishi M, Ger El, Nabi AR, and Al-Mufarrej SI (2015). Immune responsiveness and performance of broiler chickens fed a diet supplemented with high levels of Chinese *Star anise* fruit (lfficiiimverum Hook. f).Journal of Animal and Veterinary Advances, 14(2): 36-42. Available at: https://medwelljournals.com/abstract/?doi=javaa.2015.36.42

Amad AA, Manner A, Wendler KP, Neumann K, and Zentek J (2011). Effects of a phytogenic feed additive on growth performance and ileal nutrient digestibility in broiler chickens. Poultry Science, 90: 2811-2816. DOI: https://www.doi.org/10.3382/ps.2011-01515

An HJ, Rim HK,Jeong HJ, Hong SH, Um JY,and Kim HM (2010). Hot water extracts of CLV vulgaris improve immune function in protein deficient weanling mice and immune cells. ImmunopharmacolImmunotoxicol, 32:585-592. DOI: https://www.doi.org/10.3109/08923971003604778

Andrade LM, Andrade CJ, Dias M, Nascimento CAO, and Mendes MA (2018). CLV and Spirulina Microalgae as Sources of Functional Foods, Nutraceuticals, and Food Supplements; an Overview. Food Processing and Technology, 6(1):144. DOI: https://www.doi.org/10.15406/mojfpt.2018.06.00144

Ayoub HF, Abdelghany MF, and El-Sayed AB (2019). Effects of Diatoms *Amphora coffeaeformis* on growth parameters, nonspecific immunity, and protection of the Nile tilapia (*Oreochromisniloticus*) to Aeromonas hydrophila infection. Egyptian Journal of Aquatic Biology and amp; Fisheries, 23(1): 413-426. Available at:www.ejabf.journals.ekb.eg

Belay A, Kato T, and Ota Y (1996). Spirulina (Arthrospira): Potential application as an animal feed supplement. International Association of Applied Algology, 7th International Conference Abstracts, p. 23. Available at: https://link.springer.com/article/10.1007/BF02178573

Belotti G, Caprariis BD, Filippis PD, Scarsella M, and Verdone N (2014). Effect of *Chlorella vulgaris* growing conditions on bio-oil production via fast pyrolysis. Biomass Bioenerg, 61: 187-195. DOI: https://www.doi.org/10.1016/j.biombioe.2013.12.011

Benmalek Y, Yahia O, Belkebir A, and Fardeau ML (2013). Anti-microbial and anti-oxidant activities of Illicium verum, Crataegusoxyacanthasspmonogyna, and Allium cepa red and white varieties. Bioengineered, 4: 244-248. DOI:https://www.doi.org/10.4161/bioe.24435.

Bhatta RS, and Sharma SR (2001). Nutrient utilization and growth performance of rabbit fed oat plant meal and tall fescue hay. Asian-Australasian Journal of Animal Science, 14:1228-1232. DOI: https://www.doi.org/10.5713/ajas.2001.1228

Bhosleac NB, Evansad LV, and Edyveanb RGJ (1993). Carbohydrate production by *Amphora coffeaeformis*, a marine fouling diatom. Biofouling, 7(1): 81-91. DOI: https://www.doi.org/10.1080/08927019309386245

Blinkova LP, Gorobets OB, and Baturo AP (2001). Biological activity of Spirulina ZhMikrobiol Epidemiol Immunobiol, Mar-Apr. 8 (2): 108-114. . 2001 Mar-Apr; (2): 114-118. Review. In this review information of Spirulina platensis (SP), a blue-green alga (photosynthesizing cyanobacterium) having diverse biological activity is presented. Due to high content of highly. ZhMikrobiol Epidemiol Immunobiol, 2: 114-118. Available at:phytomedica.co.uk/pdf/immulina_herpes.pdf

Bolu SA, and Balogun OO (2004). Effects of improved (addition of antimicrobial and antioxidant) locally produced natural vitamin premix on the performance, nutrient retention, and carcass measurements of broilers. Centre Point, 10:83-92. DOI:http://www.dx.doi.org/10.5772/58342

Borah JC (2015). Shikimic acid: a highly prospective molecule in the pharmaceutical industry. Current Science, 109: 1672-1679. DOI: https://www.doi.org/10.18520/v109/i9/1672-1679

Cannon DC, Olitzky I, and Inkpen JA (1974). Proteins. In: Clinical chemistry, principles, and techniques. 2nd edition. Harper and Row, New York, Pp. 407-421. DOI:https://www.doi.org/10.1016/0921-4488(95)00665-8.

Chaney AL, and Marbach EP (1962). Modified reagents for determination of urea and ammonia. Clinical chemistry, 8(2): 130-132. Available at:https://academic.oup.com/clinchem/article-abstract/8/2/130/5672375

ChempakamB,and Balaji S (2008). *Star Anise*. United Kingdom: CAB International, pp. 319-330. DOI:https://www.doi.org/10.21475/ajcs.20.14.03.p2209

Cinthia E, Ricardo VN, and Paulo CP (2013). Carcass yield and sensorial analysis of meat from broiler chicken fed with tilapia by products meal.Ciência e Agrotecnologia, 37(5): 451-456. DOI: http://dx.doi.org/10.1590/S1413-70542013000500009

Ding IX, Yang CW, and Yang ZB (2017). Effects of *Star anise* (Illicium verum Hook. f.), essential oil, and leavings on growth performance, serum, and liver antioxidant status of broiler chickens. Journal of Applied Poultry Research, 26(4): 459-466. DOI: https://www.doi.org/10.3382/japr/pfx014.

Doumas BT, Watson WA, and Biggs HG (1971). Albumin standard and the measurement of serum albumin with bromocresol green, Clinicachimica acta, 31: 87-96. DOI: https://www.doi.org/10.1016/0009-8981(71)90365-2

Elmasry TA, Al-Shaalan NH Tousson E, El-Morshedy K, and Al-Ghadeer A (2018). *Star anise* extracts modulation of reproductive parameters, fertility potential, and DNA fragmentation induced by growth promoter Equigan in rat tests. Brazilian Journal of Pharmaceutical Sciences, 54:217-261. DOI: https://doi.org/10.1590/s2175-97902018000117261.

El-Sayed AB, Aboulthana WM, El-Feky AM, Ibrahim NE, and Seif MM (2018). Bio and phytochemical effect of *Amphora coffeaeformis* extract against hepatic injury induced by paracetamol in rats, Molecular Biology Reports, 45:2007–2023. DOI: https://doi.org/10.1007/s11033-018-4356-8

Enwereuzoh UO, and Onyeagoro GN (2014). A novel aeration method for the preparation of algae (Dunaliella Salina) biomass for biofuel production. American Journal of Engineering Research, 3(9): 209-214. Available at: https://scholar.google.co.uk

Ertas ON, Guler T, Iftci MC, Dalkilic B, and Simsek G (2005). The effect of an essential oil mix derived from oregano, clove and anise on broiler performance. International Journal of Poultry Science, 11: 879-884.

Feldman BF, Zinkl JG, and Jain NC (2000). Schalm's Veterinary Hematology, 5th edition, Philadelphia, Pp. 21-28. Available at:https://cmc.marmot.org/Record/.b21373000

Ferdous F, Maurice D, and Scott T (2008). Broiler chickens thrombocyte response to lipo polysaccharide. Poultry Science, 87:61–63. DOI: https://www.doi.org/10.3382/ps.2007-00356

Food and Agriculture Organization (2018). News Article: World's future food security "in jeopardy" due to multiple challenges, report warns Available at: http://www.fao.org/news/story/en/item/471169/icode/

Gasparino E, Del Vesco AP, Voltolini DM, Nascimento CS, Batista E, Khatlab AS, Grieser DO, Zancanela V, and Guimar ASE (2014). The effect of heat stress on GHR, IGF-I, ANT, UCP, and COXIII mRNA expression in the liver and muscle of high and low feed efficiency female quail. British Poultry Science, 55:466-73. DOI: https://doi.org/10.1080/00071668.2014.925090

Ghazalah A, and Ali A (2008). Rosemary leaves as a dietary supplement for growth in broiler chickens. International Journal of Poultry Science, 7: 234-239. Available at:https://scialert.net/abstract/?doi=ijps.2008.234.239

Guobin C, Xiangping L, and Jin L (2011). "Temporal and spatial expression of the Pax-7 gendering chicken embryo and postnatal development, " Journal of Animal and Veterinary Advances, 10: 1785-1788.
DOI:https://www.doi.org/10.3923/javaa.2011.1785.1788

Hirahashi T, Matsumoto M, Hazeki K, Saekia Y, Uic M, and Seyaa T (2002). Activation of the human innate immune system by Spirulina: augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis* International Immunopharmacology, pp.423-434. DOI: https://doi.org/10.1016/S1567-5769(01)00166-7

Hirata T, Tanaka M, Ooike M, Tsunomura T, and Morihiko S (2000). Antioxidant activities of phycocyanobilin prepared from Spirulina platensis. Journal of Applied Phycology, 12: 435–439. Available at: https://link.springer.com/article/10.1023/A:1008175217194

Jamil ABMR, Akanda R, Rahman M, Hossain A, and Islam S (2015). Prebiotic competence of spirulina on the production performance of broiler chickens. Journal of Advanced Veterinary and Animal Research, 2: 304-309. DOI: http://dx.doi.org/10.5455/javar.2015.b94.

Jaswir I, Noviendri D, Hasrini RF, and Octavianti F (2011). Carotenoids Sources, medicinal properties, and their application in the food and nutraceutical industry. Journal of Medicinal Plants Research, 5(33): 7119-7131. DOI: https://www.doi.org/10.5897/JMPRX11.011.

Kang HK, Salim HM, Akter N, Kim DW, Kim JH, Bang HT, Kim MJ, Na JC, Hwangbo J, Choi HC et al. (2013). Effects of various forms of dietary CLV supplementation on growth performance, immune characteristics, and intestinal microflora population of broiler chickens. Journal of Applied Poultry Science, 22: 100-108. Available at: https://www.researchgate.net/publication/270080380_Effect_of_various_forms_of_dietary_Chlorella_supplementation_on_growth_performance_immune_characteristics_and_intestinal_microflora_population_of_broiler_chickens

Khan RU, Naz S, Nikousefat Z, Tufarelli V, and Laudadio V (2012). Thymus vulgaris: alternative to antibiotics in poultry feed. World Poultry Science Journal, 68: 401-408. DOI: https://www.doi.org/10.1017/S0043933912000517

Kim DK, Lillehoj HS, Lee SH, Jang SI, and Bravo D (2010). High-throughput gene expression analysis of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde, or Capsicum oleoresin. Poultry Science. 89: 68-81. DOI: https://www.doi.org/10.3382/ps.2009-00275

Kirshenbaum AS, Swindle E, Kulka M, Wu Y, and Metcalfe DD (2008). Effect of lipopolysaccharide and peptidoglycan (PGN) on human mast cell numbers, cytokine production, and protease composition. BMC Immunology, 9:1-13. Available at: https://bmcimmunol.biomedcentral.com/articles/10.1186/1471-2172-9-45.

Lauritano C, Andersen, JH , Hansen E, Albrigtsen M, Escalera L, Esposito F, Helland K, Hanssen KØ, Romano G, Ianora A (2016). Bioactivity screening of microalgae for antioxidant, anti-inflammatory, anticancer, anti-diabetes, and antibacterial activities, Frontiers in Marine Science, 3: 68. DOI: https://www.doi.org/10.3389/fmars.2016.00068

Lee SH, Karawita R, Affan A, Lee JB, Lee KW, Lee BJ, Kim DW, and Jeon YJ (2009). Potential of *Benthic diatoms*, *Achnantheslongipes*, *Amphora coffeaeformis* and Navicula sp. (Bacillariophyceae) as antioxidant sources. Algae, 24: 47-55. DOI: https://www.doi.org/10.4490/algae.2009.24.1.047

Lee SH, Lillehoj EP, Lillehoj SM, Cho DH, Park YH, Hong H, Chun K, and Park HJ (2008). Immunomodulatory properties of dietary plum on coccidiosis. Comparative Immunology, Microbiology, and Infectious Diseases, 31: 389-402. DOI: https://doi.org/10.1016/j.cimid.2007.06.005.

Lesley B, Blom JC, Timans Y, Xu B, Hunte F, Vega N, Yu J, Wang K, and Singh (2000). Novel p19 protein engages IL-12 p 40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity, 13: 715. DOI: https://wwwdoi.org/10.1016/S1074-7613(00)00070-4

Lewis SM, Bain BJ, and Bates I (2006). "Dacie and Lewis Practical Hematology", 10th edition, Philadelphia, Churchill Livingstone, pp. 335-355. Available at:https://www.worldcat.org/title/dacie-and-lewis-practical-haematology/oclc/300300127

Lillehoj HS, Kim DK, Bravo DM, and Lee SH (2011). Effects of dietary plant-derived phytonutrients on the genome-wide profiles and coccidiosis resistance in the broiler chickens. BMC Proceeding, 5: 534. Available at: https://bmcproc.biomedcentral.com/articles/10.1186/1753-6561-5-S4-S34

Manning L, Baines RN, and Chadd SA (2007). Trends in the global poultry meat supply chain. British Food Journal, 109(5): 332-342. DOI: https://doi.org/10.1108/00070700710746759

Mariey Y, Samak H, and Ibrahem M (2012). Effect of using *Spirulina platensis* algae as a feed additive for poultry diets: 1-productive and reproductive performances of local laying hens." Egypt. Poultry Science, 32(1): 201-215. Available at: https://www.semanticscholar.org/paper/EFFECT-OF-USING-SPIRULINA-PLATENSIS-ALGAE-AS-AFEED-Mariey-Samak/22d0a2870cf60cdaf497b823cc6a617b9812ca2e

Mason R (2001). Chlorella and Spirulina: Green supplements for balancing the body. Alternative and Complementary Therapies, 7(3):161-165. Available at: https://www.liebertpub.com/doi/pdf/10.1089/107628001300303691

McCracken AN, and Edinger AL (2013). Nutrient transporters: the Achilles' heel of anabolism. Trends in Endocrinology and Metabolism. Science Direct, 24: 200-208. Available at: https://doi.org/10.1016/j.tem.2013.01.002.

Mcdevitt RM, Brooker JD, Acamovic T, and Sparks NHC (2006). Necrotic enteritis; a continuing challenge for the poultry industry.

World's Poultry Science Journal, 62:221-247. https://doi.org/10.1079/WPS200593.

Mekkawy I, Mahmoud U, Moneeb R, and Sayed AED (2020). Significance assessment of *Amphoracoffeaeformis* in arsenic-induced hemato-biochemical alterations of African catfish (Clariasgariepinus), Frontiers in Marine Science, p.7. DOI: https://doi.org/10.3389/fmars.2020.00191

Mohammed AL (2014). Immunomodulatory effect of Curcuma longa in mice. Basrah Journal of Veterinary Research, 13(1): 222-234.

Mohammed GA (2008). The effect of anise and rosemary on broiler performance. International Journal of Poultry Science, 3: 243-245. DOI: https://www.doi.org/10.3923/ijps.2008.243.245

Nwanna LC (2003). Nutritional Value and digestibility of fermented shrimp head waste meal by African Catfish Claires gariepinus, Pakistan Journal of Nutritional, 2(6): 339-345.DOI: https://www.doi.org/10.1.1.487.9192and rep

Ohira H, Torii N, Aida TM, Watanabe M, and Smith RLJ (2009). Rapid separation of shikimic acid from Chinese *Star anise* (Illicium verum Hook. f.) with hot water extraction. Separation Purification Technology, 69: 102-108. DOI: https://doi.org/10.1016/j.seppur.2009.07.005.

Parnell J, and Trevor RH (2007). Reconstructing the tree of life: taxonomy and systematics of species-rich taxa. Boca Raton, 61, 3007-3011. DOI: 10.1201/9781420009538.ch17

Philbin V, Iqbal J, Boyd M, Goodchild Y, Beal JM, Bumstead KR, Young N, and Smith AL (2005). Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. Immunology, 114: 507-521. DOI: https://doi.org/10.1111/j.1365-2567.2005.02125.

Priyanka R, and Muralidharan (2014). Interferons and interferon therapy. Journal Pharmaceutical Science and Research, 6: 400-403.

Rahmani HR, and Speer W (2005). Natural additives influence the performance and humoral immunity of broilers. International Journal of Poultry Science, 4(9):713-717. Available at: http://agris.fao.org/ID=DJ2012051528

Rajput R, and Mishra AP (2012). A review of biological activity of quinazolinones. International Journal of Pharmacy and Pharmaceutical Sciences, pp. 66-70. Available at: https://pdfs.semanticscholar.org/.

Rao RR, Platel K, and Srinivasan K (2003). In vitro influence of spices and spice-active principles on digestive enzymes of rat pancreas and small intestine. Nahrung, 4: 408-412. DOI: https://doi.org/10.1002/food.200390091.

Reitman S, and Frankel S (1957). A colorimetric method for the determination of serum glutamate oxaloacetic acid and pyruvic acid transaminases. American Journal of Clinical. Pathology, (29):56-63. Available at:https://www.scirp.org/(S(i43dyn45teexjx455qlt3d2q))/reference/ReferencesPapers.aspx?ReferenceID=1528104

Ritter AMV, Ames FQ, Otani F, de Oliveria RMW, Cuman RKN, and Bersani-Amado CA (2014). Effects of anethole in Nociception experimental models. Hindawi Publishing Corporation, Evidence-Based Complementary and Alternative Medicine, Article ID 345829. P. 7. DOI:https://doi.org/10.1155/2014/345829

Salahuddin N, Elbarbary AA, and Alkabes HA (2017). Antibacterial and anticancer activity of loaded quinazolinone polypyrrole/chitosan silver chloride nanocomposite. International Journal of Polymeric Materials and Polymeric Biomaterials, 66: 307-316. DOI: https://www.doi.org/10.1080/00914037.2016.1201831

Scanes CG (2009). Perspectives on the endocrinology of poultry growth and metabolism. General and Comparative Endocrinology, 163: 24-32. DOI: https://doi.org/10.1016/j.ygcen.2009.04.013.

Selvaraj V, Armistead MY, Cohenford M, and Murray E (2013). Arsenic trioxide (As2O3) induces apoptosis and necrosis mediated cell death through mitochondrial membrane potential damage and elevated production of reactive oxygen species in PLHC-1 fish cell line, Chemosphere, 90(3): 1201-1209. DOI: https://doi.org/10.1016/j.chemosphere.2012.09.039.

Shahrajabian MH, Sun W, and Cheng Q (2019).Chinese *Star anise* and anise, magic herbs in traditional Chinese medicine and modern pharmaceutical scienceBiotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, Asian Journal of Medical and Biological Research, 5(3): 162-179. DOI: https://doi.org/10.3329/ajmbr.v5i3.43584.

Shawky SM, Fathalla SI, Zahran IS, Gaafar KM, Hussein MK, and Abu-Alya IS (2020b). The immunological stimulant effect of linseed oil and fennel oil supplemented diet on broilers. Advances in Animal and Veterinary Sciences, 8(7): 771-776.DOI | http://dx.doi.org/10.17582/journal.aavs/2020/8.7.771.776

Shawky SM, Orabi SH, and Dawod A (2020a). Effect of marjoram supplementation on growth performance and some immunological indices in broilers. International Journal of Veterinary Science, 9(2): 297-300. DOI: https://www.doi.org/10.37422/IJVS/20.026

Shu X, Liu XM, Fu CL, and Liang QX (2010). Extraction, characterization, and antitumor effect of the polysaccharides from *Star anise* Illicium verum (Hook f). Journal of Medicinal Plants Research,4: 2666-2673. DOI: https://www.doi.org/10.5897/JMPR09.438

Soltan MA, Shewita RS, and El-Katcha MI (2008). Effect of dietary anise seeds supplementation on growth performance, immune response, carcass traits, and some blood parameters of broiler chickens. International Journal of Poultry Science, 7: 1078-1088. DOI: https://www.doi.org/10.3923/ijps.2008.1078.1088

Sornplang P, Leelavatcharamas V, and Soikum C (2015). Heterophils Phagocytic Activity Stimulated by Lactobacillus salivarius L61 and L55 Supplementation in Broilers with Salmonella Infection. Asian-Australasian Journal of Animal Sciences, 28(11): 1657-1661. DOI: https://www.doi.org/10.5713/ajas.15.0359.

Sri PU, Leelavathi V, Sree VN, and Kumar MA (2015). Antihelmenthic and antimicrobial activity of green synthesized silver nanoparticles from Illicium Verum Hook. F. fruit. IOSR Journal of Pharmacy and Biological Sciences, 10: 61-65.Available at: https://www.semanticscholar.org/paper/Antihelmenthic-And-Antimicrobial-Activity-Of-Green-Sri-Leelavathi/9a8a70d393f464ee18e19d529c95f0dfbdf42e1c

Sugiharto S, Yudiarti T, Isroli I, and Widiastuti E (2018). Effect of feeding duration of Spirulina platensis on growth performance, hematological parameters, intestinal microbial population, and carcass traits of broiler chicks South African. Journal of Animal Science, 48(1): 98-107. DOI: https://www.doi.org/10.4314/sajas.v48i1.12.

Sung YY, Kim YS, and Kim HK (2012a). Illicium verum extract inhibits TNF-n-and IFN-y-induced expression of chemokines and cytokines in human keratinocytes. Journal Ethnopharmacol, 144: 182-189. DOI: https://www.doi.org/10.1016/j.jep.2012.08.049

Sung YY, Yarig WK, Lee AY, Kim DS, Nho KJ, Kim YS, and Kim HK (2012b). The topical application of an ethanol extract prepared from Illicium verum suppresses atopic dermatitis in NC/Nga mice. Journal Ethnopharmacol, 144: 151-159. DOI: https://doi.org/10.1016/j.jep.2012.08.042.

Tavares-Dias M, and Moraes FR (2003). Hematological evaluation of Tilapia rendalli Boulenger, 1896 (Osteichthyes: Cichlidae) captured in a fee fishing farm from Franca, So Paulo, Brasil (in Portuguese). Bioscience Journal, 19: 103-110. Available at:https://agris.fao.org/agris-search/search.do?recordID=BR2003004654

Trinchieri G (1994). Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes, Blood. 84: 4008. Available at:https://www.thermofisher.com/antibody/product/Human-IL12-Recombinant-Protein/PHC1124.

Young DS (2001). Effects of disease on the clinical lab. Tests, 4th edition. AACC press, 48 (4): 682-683. Available at:https://www.scirp.org/(S(351jmbntvnsjt1aadkposzje))/reference/ReferencesPapers.aspx?ReferenceID=1272186.

Zhang GF, Yang ZB, Wang Y, Yang WR, Jiang SZ, and Gai GS (2009). Effects of ginger root (Zingiber officinale) processed to different particle sizes on growth performance, antioxidant status, and serum metabolites of broiler chickens. Poultry Science, 88: 2159-2166. DOI: https://doi.org/10.3382/ps.2009-00165.

Zhao R, Muehlbauer E, Decuypere E, and Grossmann R (2004). Effect of genotype–nutrition interaction on growth and somatotropic gene expression in the chicken. General and Comparative Endocrinology, 136: 2–11. DOI: https://www.doi.org/10.1016/j.ygcen.2003.11.009

Zhou BG, Wang S, Dou TT, Liu S, Li MY, Hua, RM, Li SG and Lin HF (2016). Aphicidal activity of Illiumverum fruit extracts and their effects on the acetylcholinesterase and glutathione S-transferases activities in Myzuspersicae (Hemiptera: Aphididae). Journal of Insect Science, 16: 1-7. DOI: https://doi.org/10.1093/jisesa/iev163

Zhou CZ, Meyer P, Quevillon-Cheruel S, Li De La Sierra-Gallay I, Collinet B, Graille M., Blondeau K, François JM, and Sorel I (2005). Crystal structure of the YML079w protein from Saccharomyces cerevisiae reveals a new sequence family of the jelly-roll fold. Protein Science, 14(1):209-2015. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2253319/

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 643-648, December 25, 2020

Research Paper, PII: S2322455X2000073-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.73

Seroprevalence of Avian Influenza Virus Subtype H5 among Poultry Workers of Central Traditional Markets in Indonesia

Dina Novitasari and Chairul Anwar*

Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia.

*Corresponding author`s Email: chairulhisto@gmail.com; ORCID: 0000-0002-3309-5291

Received: 04 Nov. 2020
Accepted: 18 Dec. 2020

ABSTRACT

Avian Influenza (AI) has been spread rapidly in almost all the provinces of Indonesia by the end of 2007, and it has become endemic. Avian Influenza viruses can be infecting to human from direct or indirect contact with the infected or dead poultry, and a visit to the wet poultry market in the neighborhood. Seroprevalence studies can be used to identify the clinical key, epidemiological studies, and the spread of AI viruses in humans. The aim of present study was to investigate the seroprevalence of Avian Influenza (AI) virus, subtype H5 among poultry workers at the central traditional market in industrial. To meet the mentioned demand, 26 blood samples were collected from the poultry workers via the median cubital vein. The antibody titer was examined using Hemagglutination Inhibition (HI) assay using H5 antigen from duck licensed under A/Dk/Indonesia/AU-78/12 (H5N1) and three kinds of red blood cells taken from horse, chicken and guinea pig. The serum samples were added with Receptor Destroying Enzyme (RDE) with a ratio of 3:1 (v/v) for an overnight, and pretreated with 10% of red blood cells before the HI assay was conducted. Based on the findings, it can be concluded that the percentage of seroprevalence of Avian Influenza (AI) virus, subtype H5 among poultry workers at central traditional market was 0%. Thirteen samples showed a negative result of the HI test. All of the workers' blood serum obtained less than 2^4 antibody titer from the HI test. As the results showed, the research on the poultry workers in the traditional market was carried out, and it obtained negative results; all the workers were not infected with the Avian Influenza virus. In other words, Avian Influenza is not meaningful in poultry farm workers in Sidoarjo suburb traditional farms.

Key words: Avian Influenza, Poultry workers, Seroprevalence, Traditional market.

INTRODUCTION

Avian influenza (AI), H5N1 is one of the respiratory diseases which commonly affected poultry caused by an influenza virus from the *Orthomyxovirida* family; the virus can also be transmitted to human (Al-Natour and Abo-Shehada, 2005; Wang and Alexander, 2021). The epidemic spread of avian influenza, H5N1 in Indonesia occurred in Java in August 2003 affected birds, which led to the spread of the disease to other areas of south east Asia (Gutiérrez et al., 2009). By the end of 2005, the disease was considered endemic in parts of Java, Sumatra, Kalimantan, Sulawesi, and Bali (Santhia et al., 2009). The first zoonotic transmission was confirmed in western Java in July 2005 (Wiyono et al., 2004; Takano et al., 2009). From then on, a total of 199 human H5N1 cases have been reported, in which 167 were fatal (WHO, 2014). Avian Influenza virus in Indonesia is attributed to H5N1 virus clade 2.1.3 and 2.3.2 (WHO, 2014).

Many researchers have been investigating the spread of AI virus on livestocks, poultry workers, farmers, and their family. Based on the previous study, it was found that influenza A viruse (H5N1) were spread among pigs, and gradually adapted themselves to recognize human-type receptors, affecting farmers, workers, and their families in the farm area to have a great risk of being infected or contaminated with the new viruses (Frederika et al., 2013; Lailirahmawati et al., 2015). The fatality rate of H5N1 was less than the frequent rate reported more than 50%. Although it is impossible to determine H5N1 fatality rate accurately, it could be assumed that 1% to 2% infection rate would translate a large amount of infection in exposed (Wang et al., 2012).

Avian Influenza viruses may be transmitted to human, through direct or indirect contact with the infected or dead poultry, and a visit to wet and non-cleaned poultry markets. The viruses are normally prevented or at least controlled by an immune defense; If the viruses

To cite this paper: Novitasari D and Anwar Ch (2020). Seroprevalence of Avian Influenza Virus Subtype H5 among Poultry Workers of Central Traditional Markets in Indonesia. *J. World Poult. Res.*, 10 (4): 643-648. DOI: https://dx.doi.org/10.36380/jwpr.2020.73

transferred in invading the body and overcoming the immune defenses, they may cause the disease. The viruses will survive and have sufficient time to replicate and transmit the progeny to their host (Xu et al., 2009). Seroprevalence studies have been conducted in Thailand, Cambodia, Vietnam (Dung et al., 2014), and Indonesia (Robert et al., 2010) to evaluate the clinical and epidemiological factors, and other key points related to human influenza A infections (H5N1) in 2011 (Dung et al., 2014).

The researcers were interested to investigate the sero-prevalence of Avian Influenza (AI) virus subtype H5 among poultry workers at a central traditional market in an industrial city in suburb of Sidoarjo. Therefore, the purpose of current study was to investigate the seroprevalence of Avian Influenza (AI) virus subtype H5 whose main habitual activities revolve around poultries in an industrial city's and especially in its central traditional markets.

Ethical approval

The Universitas Airlangga Ethics Committee, Airlangga, approved the employed protocol after the agreement of local health authorities for all participants.

MATERIALS AND METHODS

Collection and the storage procedure of poultry workers' blood serum and red blood cells of horse, guinea pig, and chicken

The blood sample of the workers who worked in the poultry market was taken using an incidental sampling method (Mohsin, 2016).

This research was conducted in a Biosafety Level-2 (BSL-2) laboratory in Avian Influenza Research Center (AIRC), Universitas Airlangga of Indonesia. Implementation of the present research was carried out from February to April 2017. The materials that were used in this research were 26 blood serums from the workers in Larangan Sidoarjo traditional market. The experimental laboratory components were including; duck's AI antigen subtype H5 A/Dk/Indonesia/AU-78/12 (H5N1) from Avian Influenza Research Center (AIRC), 1% and 10% horse Red Blood Cell (RBC), 0.5% and 10% chicken Red Blood Cell (RBC), 0.75% and 10% guinea pig Red Blood Cell (RBC), Phosphate Buffered Saline (PBS) solution, Receptor Destroying Enzyme (RDE) (II) "Seiken" (Denka Seiken Co., Ltd, Tokyo, Japan), physiological saline 0.9%, and alcohol 70%.

Red blood cells used for Hemagglutinin (HA) and Hemagglutinin Inhibition (HI) assay in the current research were taken from horse, chicken, and guinea pig which were collected from cephalic (horse), brachial (chicken), and intracardiac vein (guinea pig) using a syringe, and then were placed into a vacutainer tube with Ethylene Diaminetetra Acetic Acid (EDTA). The preparations of packed RBCs were first started by carefully collecting 3-5 ml blood to be placed into the conical tube by the volume of 15 ml, then filled with cold PBS, and mixed gently by inversion. The mix of RBCs and PBS were centrifuged at 3000 rpm for 5 minutes at 4^{o}C, in which the supernatant was aspirated carefully to not disturb the pellet of RBCs. The conical tube was filled gently with cold PBS again, and the process was repeated carefully for three PBS washes to prevent hemolysis.

On the other hand, Receptor Destroying Enzyme (RDE) treatment was given to remove nonspecific virus inhibitor, which would cause a false positive result in HI assay. First, a vial of RDE (II), Seiken, was reconstituted with 20 ml of 0.9% saline. Three parts of RDE were added to one part of serum (e.g. 20 µl serum + 60 µl RDE), then vortexed for homogenization. The RDE-serum mixtures were incubated at 37^{o}C for 18-20 hours (overnight) in a dry bath, and then heated in a 56^{o}C dry bath for 30 minutes to inactivate the RDE. Following after, the serum was allowed to cool in a room temperature. The 10 ml of RBCs in blood samples of horse, chicken or guinea pig was absorbed (packed RBCs) according to the procedure to remove the nonspecific agglutinin (WHO, 2013). One part of RBCs 10% was combined with one part of treated serum (e.g. 20 µl serum + 60 µl RDE + 80 µl RBCs 10%). It was mixed thoroughly and incubated at room temperature for 30 minutes, then inverted gently to be mixed every 10 minutes. The treated serums were centrifuged at 2000 rpm for 10 minutes at 4^{o}C to pellet the erythrocytes. The serum was decanted and placed in another Eppendorf to be tested in HI assay, then the erythrocytes pellet was discarded (WHO, 2013; Born et al., 2019).

Hemagglutinin assay and hemagglutinin inhibition assay with red blood cells of the horse, chicken, and guinea pig

The blood samples were taken from 24 male animals contained 4 horses, 12 chickens, and 8 guinea pigs and Hemagglutinin (HA) assay was done. The determination of HA titers of AI antigen subtype H5 (separated from animals' blood) was carried (IDVet Kit, ID Screen® Influenza H9 Elisa Antibody kit, France). The concentrate

of red blood cells was 1%, and it was put on "V-bottom" microplate (Wang et al., 2014). First, 50 µl of PBS were added to 12 wells in rows A and B (control RBC). Then, 50 µl of the antigen added to well A1. Serial of two-fold dilutions were made by transferring 50 µl from well A1 sequentially through well A12, then 50 µl were discarded from well A12. Fifty µl of 1% suspension of each kind of RBC was added to all wells in rows A and B. The plate was tapped gently to be mixed and covered with plastic wrap, then incubated at room temperature for 60 minutes. After that, the haemagglutination assay (HA) titer was read by tilting the plate at 45^o to 60^o angle. The settled RBCs in row B (control RBC) would start running, and formed a teardrop-shape due to the gravity, and the result was positive if the erythrocytes formed a diffuse film. Antigen titer was the highest dilution of virus that still caused complete hemagglutination (Born et al., 2019).

The standard working dilution of virus must have an HA titer of eight hemagglutination units (HAU) per 50 µl (as same as four HAU per 25 µl). If the working antigen did not have an HA titer of eight HAU per 50 µl, it must be adjusted accordingly to the titer of antigen by diluting the virus with PBS to decrease the number of HAU. For instance, prepared three ml of eight HAU per 50 µl from 2^4 HA titer was done by adding 1.5 ml antigen with 1.5 ml PBS. Therefore, the diluted viruses that contained eight HAU per 50 µl (or four HAU per 25 µl) must be verified by performing a second HA test/retitration (WHO, 2013). The retritration or back titration was a procedure to verify the correct units of hemagglutinin. The retitration process was carried out for each kind of red blood cells.

Besides, HI assay was also investigated for each kind of RBCs from horse, chicken, and guinea pig. Hemagglutinin assay in this research was used RBCs 1% and "V-bottom" microplate for each type of RBCs source was applied respectively. First, 25 µl of PBS were added to the well number 1 to 12. Then, 25 µl of treated serum was added to the well number 1, and serial of two-fold dilutions were made by transferring 25 µl from the first well to the well number 10. The final 25 µl were discarded. After that, 25 µl of standardized antigens containing four HAU were added to all wells containing the serum (except well number 11 and 12), and 25 µl of PBS was added to all wells in column number 11 to be used as RBC controls and all wells in column number 12 for serum control. The plate was tapped gently to be mixed and covered with plastic wrap, then incubated at room temperature for 30 minutes.

After that, 50 µl of 1% horse RBC suspension was added to each well. The plate was tapped gently to be mixed and covered with plastic wrap, then incubated at room temperature for 60 minutes. The HI titers of the serums were recorded after the 60 minutes of incubation by tilting the plate at a 45^o to 60^o angle. The settled RBCs in column number 11 should start pulling or running, and they formed a teardrop-shape due to the gravity. Antibody titer is the reciprocal of the antiserum's highest dilution that produces complete hemagglutination inhibition. Positive antibody titer is a sample that has higher or equal titer with 2^4 (15,18). The data from HI assay was presented descriptively by calculating the percentage of the antibody of AI virus subtype H5 among the workers in the poultry market.

RESULTS

Collection and storage procedure of the workers' blood serum, red blood cells of horse, guinea pig, and chicken

Based on the result of present study, 13 samples have been collected from blood serums of the workers at the Larangan traditional market in Sidoarjo, Indonesia. Red blood cells that were used for HA and HI assay in this research were from the horse, chicken and guinea pig. They were collected from the cephalic (horse), brachial (chicken), and intracardiac (guinea pig) veins. Present study succeeded in taking the samples according to the criteria of the existing procedures (WHO, 2013). All samples used in the current study were feasible and appropriate for the HA and HI test. All samples of red blood cells were collected; HA and HI assays were performed, and the data was analyzed and presented descriptively.

Hemagglutinin assay and hemagglutinin inhibition assay with the red blood cells of horse, chicken, and guinea pig

Based on the result of this study, the researchers used three different kinds of RBCs in HI test, which were from horse, guinea pig, and chicken to be compared and given a more sensitive result to detect human AI, and AI subtype H5 antigen from duck A/Dk/Indonesia/AU-78/12 (H5N1). From 13 serum samples, any evidence of positive antibodies titer was found against AI subtype H5 among the workers in the central traditional market in the industrial city of Sidoarjo, Indonesia, therefore the seroprevalence was 0% (Table 1).

Table 1. The results of hemagglutination inhibition test for the workers on avian influenza subtype H5 using RBCs of horse, guinea pig, and chicken

No.	Serum code	RBCs			Statement
		Horse	Guinea pig	Chicken	
		HI test result (antibody titer)			
1	LSR 1	$< 2^4$	$< 2^4$	$< 2^4$	Negative
2	LSR 2	$< 2^4$	$< 2^4$	$< 2^4$	Negative
3	LSR 3	$< 2^4$	$< 2^4$	$< 2^4$	Negative
4	LSR 4	$< 2^4$	$< 2^4$	$< 2^4$	Negative
5	LSR 5	$< 2^4$	$< 2^4$	$< 2^4$	Negative
6	LSR 6	$< 2^4$	$< 2^4$	$< 2^4$	Negative
7	LSR 7	$< 2^4$	$< 2^4$	$< 2^4$	Negative
8	LSR 8	$< 2^4$	$< 2^4$	$< 2^4$	Negative
9	LSR 9	$< 2^4$	$< 2^4$	$< 2^4$	Negative
10	LSR 10	$< 2^4$	$< 2^4$	$< 2^4$	Negative
11	LSR 11	$< 2^4$	$< 2^4$	$< 2^4$	Negative
12	LSR 12	$< 2^4$	$< 2^4$	$< 2^4$	Negative
13	LSR 13	$< 2^4$	$< 2^4$	$< 2^4$	Negative

Antibody titers might be regarded as positive if there is inhibition at a serum dilution of 1/16 or $\geq 2^4$ (OIE, 2014). LSR is a code for coding samples.

DISCUSSION

The purpose of the current study was to investigate the seroprevalence of Avian Influenza (AI) virus, subtype H5 among the workers in the central traditional poultry market of Sidoarjo, Indonesia. According to this research, none of the sample had a positive result (0%), nor indicated the existence of AI virus even though previous investigation which revealed that the wild birds around Larangan traditional market in Sidoarjo were infected with AI virus H5N1 (Poetranto et al., 2011). Other previous research also reported that there was a report of AI virus subtype H5 in Larangan traditional market of Sidoarjo (Frederika et al., 2013; Lailirahmawati et al., 2015). Therefore, the researchers assumed that the zero seroprevalence might be due to no avian-to-human transmission yet in the central traditional market of Larangan, Sidoarjo, Indonesia.

Avian-to-human transmission is limited and rare because the species barrier is quite strong to accommodate the transmission of H5N1 virus. Through the use of lectins Maackia amurensis agglutinin (MAA) for *SAα2,3Gal* receptors and Sambucus nigra agglutinin (SNA) for SAα2,6Gal receptors, it was discovered that avian and human influenza viruses typically have a different SA-binding preference. Therefore, host range distribution is strongly restricted by the receptor specificity due to the surface antigen of hemagglutinin (HA) protein. Human influenza viruses bind preferentially to *SAα2,6Gal* receptors which is dominantly located on epithelial cells in the upper respiratory tract of human (i.e. H1N1 and H3N2 viruses), but H5N1 viruses bind preferentially to SAα2,3Gal receptors, whereas it is predominantly located in the low respiratory tract of humans such as type II pneumocytes, alveolar macrophage, and non-ciliated cuboidal epithelial cells in terminal bronchioles. The result of this condition was the no replication of the virus due to the failure of the virus to recognize and attach to the receptors. Hence, this different distribution of the receptors might limit the H5N1 virus to infect human easily, moreover through human-to-human transmission. Zoonotic transmission of H5N1 might only occur when the virus mutated and acquired the ability to recognized SAα2,6Gal receptors besides SAα2,3Gal receptors (Shinya et al., 2006; De Graaf and Fouchier, 2014).

The negative result might also be the cause of the small number of exposures or contact with the virus. Even though poultry workers often make a contact with the dead or infected chicken or more intensive poultry exposure, and are expected to have a higher risk of infection due to the lack of preventive measures and healthy environment, it does not mean that the poultry workers must have positive antibody against Avian Influenza subtype H5 (Indriani et al., 2010; Huo et al.,

2012). besides poultry workers that are prone to AI infection, others are people who visit the wet market. However, the people do not have constant exposure or direct contact with the infected poultry. Despite of that, a lot of AI-infected cases of people who visit the wet market were acute and fatal (To et al., 2012). This might happen when the people were exposed to a high amount of virus, but did not have a good body condition, which led to a bad immune response. Avian influenza A (H5N1) in humans differs in multiple ways from the influenza as a human virus, including the routes of transmission, clinical severity, pathogenesis, and response to treatment (Kumar et al., 2018).

The result of negative inhibition test occurred is the HI reference reagent as antigen was not antigenetically related to the serum (Kumar et al., 2018). Avian Influenza virus is an RNA virus which can easily mutate. Therefore, there was a possibility where the virus was mutating into a new form and structure. The serums which the researcher used were also mostly from the poultry workers at Larangan traditional market in which the majority of the tenants sell chicken, while the antigen for this research's HI assay was using antigen originated from the duck. Furthermore, the clade of the Avian Influenza subtype H5 antigen used was clade 2.3.2, while the highest cumulative number of avian-to-human transmission for H5NI cases and death in Indonesia was caused by Avian Influenza A subtype H5 clade 2.1.3 (Yupiana et al., 2010). These differences between species and the HA phylogeny also might be contributed to the negative result. Another reason that might also affect the results was that the antigen was taken from avian influenza outbreak in 2012. Therefore, there was a possibility that the virus circulating at Larangan traditional market in Sidoarjo was not the same as the one in the outbreak in 2012. Giving interventions directly in poultry sector was the most effective way to prevent human HPAI (Yupiana et al., 2010).

This study still had some limitations such as the lack of observations that can complement the results of research on the biological characteristics of the condition of poultry workers who contracted the AI virus, no microscopic observations of blood cells infected with the AI virus, and comparison of the seropositive clade type was not done. However, the results of the current study have provided additional information that poultry workers in the central traditional market of Sidoarjo were not infected with the Avian Influenza virus. Poultry workers in the market need to be given further precautions and safeguards against AI viruses. This research can be useful for veterinarians, practitioners, breeders, poultry workers, scientists, and livestock entrepreneurs as a prevention towards the poultries from spreading the AI virus.

CONCLUSION

Based on results of the research, it can be concluded that the percentage of seroprevalence of Avian Influenza (AI) virus subtype H5 among poultry workers at the central traditional market in Sidoarjo was 0%, which means that none of the workers tested were contaminated with AI virus subtype H5.

DECLARATION

Competing interests

All authors have no conflict of interest to declares

Author`s contribution

Dina Novitasari and Chairul Anwar had similar roles in conduction and collection of samples and data, as well as analysis of data. Both authors checked and approved the final version of the manuscript.

REFERENCES

Al-Natour MQ, and Abo-Shehada MN (2005). Sero-prevalence of avian influenza among broiler-breeder flocks in Jordan. Preventive Veterinary Medicine, 70(1–2): 45-50. DOI: https://doi.org/10.1016/j.prevetmed.2005.02.009

Born S, Dörfel MJ, Hartjen P, Haschemi Yekani SA, Luecke J, Meutsch JK, Westphal JK, Birkelbach M, Köhnke R, Smeets R et al. (2019). A short-term plastic adherence incubation of the stromal vascular fraction leads to a predictable GMP-compliant cell-product. BioImpacts, 9(3): 161-172. DOI: https://doi.org/10.15171/bi.2019.20

De Graaf M, and Fouchier RAM (2014). Role of receptor binding specificity in influenza A virus transmission and pathogenesis. EMBO Journal, 33(8): 823-841. DOI: https://doi.org/10.1002/embj.201387442

Dung TC, Dinh PN, Nam VS, Tan LM, Hang NLK, and Thanh LT (2014). Seroprevalence survey of avian influenza A (H5N1) among live poultry market workers in northern Vietnam, 2011. Western Pacific Surveillance and Response Journal, 5(4): 21. PMID: 25685601

Frederika E, Mareta A, Poetranto D, Wulandari L, Setyoningrum RA, and Setyowati LL (2013). Identification of influenza viruses in human and poultry in the area of larangan wet market sidoarjo-east java, Indonesia. Indonesian Journal of Tropical and Infecous Disease, 4(4): 30-34. DOI: http://dx.doi.org/10.20473/ijtid.v4i4.230

Gutiérrez R A, Naughtin MJ, Horm SV, San S, and Buchy P (2009). A(H5N1) Virus evolution in South East Asia. Viruses, 1(3): 335-361. DOI: https://doi.org/10.3390/v1030335

Huo X, Zu R, Qi X, Qin Y, Li L, and Tang F (2012). Seroprevalence of avian influenza A (H5N1) virus among poultry workers in Jiangsu Province, China: an observational study. BMC Infectious Diseases, 12: 93. DOI: https://doi.org/10.1186/1471-2334-12-93

Indriani R, Samaan G, Gultom A, Loth L, Indryani S, and Adjid R (2010). Environmental sampling for avian influenza virus A (H5N1) in live-bird markets, Indonesia. Emerging Infectious Diseases, 16(12): 1889-1895. DOI: https://doi.org/10.3201/eid1612.100402

Kumar B, Asha K, and Khanna M (2018). The emerging influenza virus threat: status and new prospects for its therapy and control. Archives of Virology, 163: 831-844. DOI: https://doi.org/10.1007/s00705-018-3708-y

Lailirahmawati I (2015). Isolasi Dan Identifikasi Virus Ai (Avian Influenza) Subtipe H5 Pada Ayam Sakit Yang Diperdagangkan Pada Pasar Larangan Sidoarjo. Universitas Airlangga, Airlanga, Pp. 1-6. Available at: http://repository.unair.ac.id/21114/4/4.%20BAB%201%20PENDAHULUAN.pdf

Mohsin A (2016). A Manual for Selecting Sampling Techniques in Research. University of Karachi, Munich Personal RePEc Archive Publication, Munich, Pp. 9-14. Available at: https://mpra.ub.uni-muenchen.de/70218/1/

OIE (2014). Terrestrial manual. Avian Influenza, P. 4. Available at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf

Poetranto ED, Yamaoka M, Nastri AM, Krisna LAW, Rahman MH, and Wulandari L (2011). An H5N1 highly pathogenic avian influenza virus isolated from a local tree sparrow in Indonesia. Microbiology and Immunology, 55(9): 666-672. DOI: https://doi.org/10.1111/j.1348-0421.2011.00361.x

Robert M, Ry D, Holle VB, Setiawaty V, Pangesti KNA, and Sedyaningsih E (2010). Seroprevalence of avian influenze A H5N1 among poultry farmers in rural Indonesia 2007. Southeast Asian Journal of Tropical and Medical Public Health, 41(5): 1095-1103. Available at: https://linkinghub.elsevier.com/retrieve/pii/S1201971208004633

Santhia K, Ramy A, Jayaningsih P, Samaan G, and Gde A (2009). Avian influenza A H5N1 infections in Bali province , Indonesia: a behavioral , virological and seroepidemiological study. Influenza Other Respiratory Viruses, 3: 81-89. DOI: https://dx.doi.org/10.1111%2Fj.1750-2659.2009.00069.x

Shinya K, Ebina M, Yamada S, Ono M, Kasai N, and Kawaoka Y (2006). Avian flu: influenza virus receptors in the human airway. Nature, 440(7083): 435. DOI: https://doi.org/10.1038/440435a

Takano R, Nidom CA, Kiso M, Muramoto Y, Yamada S, and Sakai-Tagawa Y (2009). Phylogenetic characterization of H5N1 avian influenza viruses isolated in Indonesia from 2003–2007. Virology, 390(1): 13-21. DOI: https://doi.org/10.1016/j.virol.2009.04.024

To KKW, Ng KHL, Que TL, Chan JMC, Tsang KY, Tsang AKL Chen H, and Yuen KY (2012). Avian influenza A H5N1 virus: a continuous threat to humans. Emerging Microbes & Infections, 1(9): e25. DOI: https://doi.org/10.1038/emi.2012.24

Wang G, Zhang,T, Li X, Jiang Z, Jiang Q, Chen Q, Tu X, Chen Z, Chang J, Li L et al. (2014). Serological evidence of H7, H5 and H9 avian influenza virus co-infection among herons in a city park in Jiangxi, China. Scientific Reports, 4: 6345. DOI: https://doi.org/10.1038/srep06345

Wang L, and Alexander CA (2021). COVID-19 compared with other viral diseases: novelties, progress, and challenges. Electronic Journal of General Medicine, 18(1): 265. DOI https://doi.org/10.29333/ejgm/8575

Wang TT, Parides MK, and Palese P (2012). Seroevidence for H5N1 influenza infections in humans: meta-analysis. Science, 335(6075): 1463. Availble at: https://doi.org/10.1126/science.1218888

WHO (2013). Laboratory procedures. Serological detection of avian influenza A (H7N9) virus infections by modified horse red blood cells haemagglutination-inhibition assay. 20 December 2013- Geneva: WHO, pp. 1-12 Availble at: https://www.who.int/influenza/gisrs_laboratory/cnic_serological_diagnosis_hai_a_h7n9_20131220.pdf

WHO (2014). Antigenic and genetic characteristics of zoonotic influenza viruses and development of candidate vaccine viruses for pandemic preparedness. Weekly Epidemiological Record, Available at: https://www.who.int/influenza/vaccines/virus/characteristics_virus_vaccines/en /

Wiyono A, Indriani R, Dharmayanti N, and dan Darminto RD (2004). Isolasi dan karakterisasi virus HPAI subtipe H5 dari ayam asal wabah di Indonesia. JITV, 9(3): 61-71. Available at: http://repository.pertanian.go.id/bitstream/handle/123456789/3147/Isolation%20and%20characterization%20of%20virus%20of%20highly%20pathogenic%20avian%20influenza%20H5%20subtype%20of%20chicken%20from%20outbreaks%20in%20Indonesia.pdf?sequence=1&isAllowed=y

Xu C, Dong L, Xin L, Lan Y, Chen Y, and Yang L (2009). Human avian influenza A (H5N1) virus infection in China. Science in China Series C: Life Sciences, 52(5): 407-411. DOI: https://doi.org/10.1007/s11427-009-0067-7

Yupiana Y, Vlas SJ De, Adnan NM, and Hendrik J (2010). Risk factors of poultry outbreaks and human cases of H5N1 avian influenza virus infection in West Java province, Indonesia. International Journal of Infectious Diseases, 14(9): 800-805. DOI: http://dx.doi.org/10.1016/j.ijid.2010.03.014

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(4): 649-661, December 25, 2020

Research Paper, PII: S2322455X2000074-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.74

Characterization and Analysis of the Major Structural Protein Genes of the Recently Isolated Avian Infectious Bronchitis Virus in Egypt

Nahed Yehia*, Dalia Said and Ali M. Zanaty

Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Agriculture Research Center, Dokki, Giza 12618, Egypt

*Corresponding author's Email: nahedyehia@yahoo.com, ORCID: 0000-0002-2823-6467.

Received: 09 Nov. 2020
Accepted: 21 Dec. 2020

ABSTRACT

Infectious Bronchitis Virus (IBV) is a severe infectious disease affecting chickens and causing serious economic loss. Although several studies have been conducted to characterize HVRs-S1 (Hyper-Variable Regions of Spike 1 gene) in Egypt, few of which aimed to characterize the major structural protein genes. In the present study, the genetic characterization of the major structural protein genes was carried out in 10 isolates selected from six governorates in 2019. Phylogenetically, the S1 gene was clustered into genotype GI-23 (variant II), with seven viruses that were clustered into Egy/Var II occurring in two subgroups (I, II) when aligned with previously isolated Egyptian strains. It had a specific character of 40 Amino Acids (AA) mutations except for IBV/EG/CV32/2019, which had 50 AA mutations, specifically in HVRs regions (HVRI, II, and III). The other three strains were clustered into Egy Var I with 17 AA mutations except IBV/EG/F859/ 2019, which had 15 AA mutations, compared to IBV/CU/4/2014 reference strain. The examined isolates had an additional glycosylation site at position 280 and one was missing at position 139 with the exception of two strains that only had an additional one, compared to IBV/CU/4/2014. The viruses in this study differed genetically from various vaccine seeds in the range of 69-83%. The Nucleocapsid, genetically characterized in the group of variant II (Egy/Var II) and the glycoprotein membrane genes genetically characterized in the variant group in a new sub-group with 11 and 9 AA mutations, respectively. The recombination event was only detected in the S1 gene in two isolates of IBV/EG/CV32/2019 and IBV/EG/F859/2019 from D274 and QX, respectively. In this regard, it is important to conduct continuous surveillance, pathogenicity study, and vaccine efficacy evaluation.

Keywords: Characterization, Infectious bronchitis virus, Major structure protein, Matrix, Nucleoprotein, Spike

INTRODUCTION

Infectious bronchitis virus (IBV) is a serious viral disease affecting poultry and causing high economic loss worldwide (Cavanagh et al., 2007; Milek et al.,2018). It is caused by avian IBV, a member of the *Coronaviridae* family, the 99+*Coronavirinae* subfamily and genus *Gammacoronavirus* (Carstens, 2009). It affects the respiratory, reproductive, and renal systems of all ages in different ways and increases the exposure to other pathogen infections (Cavanagh et al., 2007; Jackwood, 2012).

The IBV genome is a single-stranded and positive-sense (Masters and Perlman, 2013). The genome consists of 5 ′-UTR-1a-1b-S-3a-3b-E-M-4b-4c-5a-5b-N-6b-UTR-poly (A) tail-3′ (Brierley et al., 1989) encodes major structural proteins and non-structural protein. The major structural protein is composed of glycoprotein Spike (S), Nucleocapsid (N) protein, glycoprotein Membrane (M), and protein Envelope (E) (Thor et al., 2011; Wang et al., 2017)

The S glycoprotein is a surface protein cleaved into two subunits of S1 and S2. The S1 subunit constitutes the highly variable globular head, which was responsible for the serotype and virus neutralization (Cavanagh et al., 1992). In addition, it contains a receptor binding site that is important for tissue tropism (Ammayappan et al., 2009). The N protein plays a significant role in the replication and assembly of the virus (Lai and Holmes, 2001). The S1 and N are the key genes for determining pathogenicity, evolution, and diversity of IBV (Lee et al., 2003; Ammayappan et al., 2009). The M protein is mainly responsible for the viral assembly process (Corse and Machamer, 2003). Several IBV serotypes and genotypes

with minimal cross-protection were found around the world. The IBV evolves rapidly in nature through the substitution, insertion, deletion and/or recombination of different genes (Jackwood, 2012; Hewson et al., 2014; Hassan et al., 2019). Thus, the new highly virulent viruses emerge with minimal cross-protection leading to vaccination failure (Cavanagh et al., 2007; Jackwood, 2012). Multiple serotypes and genotypes of IBV were found in Egypt and co-circulated in the field (Abdel-Moneim et al., 2002; Abdel-Moneim et al., 2012). Depending on the complete S1 sequence, Valastro et al (2016) grouped the Egyptian variant strains into the GI-23 lineage. An Egyptian variant I strain was identified in various poultry farms in 2001 (Abdel-Moneim et al., 2002). In 2011, the new variant (Egyptian variant II) was detected in both vaccinated and non-vaccinated flocks causing severe outbreaks (Abdel-Moneim et al., 2012). The Egyptian variant II differed from the classical vaccine H120 and Ma5 used in Egypt (Abd El Rahman et al., 2015). In 2012, an upgrade of the vaccines was introduced to control the outbreak in Egypt using "variant" vaccine strains 1/96, 4/91, CR88, and D274 (Abozeid et al., 2017). With this background in mind, the present study aimed to investigate the variability of major IBV structural protein genes in Egypt (S1, N, and M) during 2019 using 10 isolates from different governorates and evaluate current control measures in the field.

MATERIALS AND METHODS

Ethical approval

The present study did not work on animals or human participants directly.

Isolation information

In the present study, ten IBV isolates from ten infected chicken farms from six different governorates were isolated in 2019 in nine to 11-days-old Specific Pathogenic Free Embryonated Chicken Egg (SPF-ECE) in the allantoic fluids and then the allantoic fluid was collected after 48 hours post-inoculation and stored at -80 °C (Li et al., 2012). The S1, N, and M genes were sequenced and published by the National Center for Biotechnology Information (NCBI) with the accession number provided in Table1.

Polymerase chain reaction and sequencing of S1, N, and M genes from IBV isolates

Viral RNA was extracted from the infected allantoic fluid of Specific Pathogen Free (SPF) eggs using a mini kit of QIAmp viral RNA (Qiagen, Hilden, Germany), as instructed by the manufacturers. The cDNA synthesis was performed using a First-Strand Synthesis System SuperScriptTM III (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The S1, N, and M genes were amplified using specific primers (Table 2) and high fidelity Phusion® DNA polymerase (Thermo Fisher Scientific, MA, USA) according to the manufacturer protocol. The amplification of the reverse transcription-Polymerase Chain Reaction (PCR) were detected by agarose gel electrophoresis. The purification was carried out using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Full-length sequencing was performed with gene-specific primers using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and the nucleotide sequence was obtained from an ABI 3500 Genetic Analyzer (Life Technologies, California, USA).

Table 1. Epidemiological data and accession number of major structural protein genes (S1, N, and M) of IBV isolates by the National Center for Biotechnology Information

	Code	collection Date	Governorate	GenBank Accession number		
				S1	N	M
1	IBV/EGY/CH/CV48/2019	2/2019	Giza	MN651560	MT085346	MT085356
2	IBV/EGY/CH/CV10/2019	1/2019	Sharqia	MN651561	MT085342	MT085359
3	IBV/EGY/CH/CV17/2019	5/2019	Behera	MN651562	MT085343	MT085358
4	IBV/EGY/CH/CV31/2019	5/2019	Sharqia	MN651563	MT085344	MT085353
5	IBV/EGY/CH/CV32/2019	3/2019	Behra	MN651564	MT085349	MT085352
6	IBV/EGY/CH/CV125/2019	1/2019	Giza	MN651565	MT085347	MT085354
7	IBV/EGY/CH/F580/2019	7/2019	Dakhlia	MN651566	MT085345	MT085361
8	IBV/EGY/CH/F564/2019	6/2019	Qaliobia	MN651567	MT085348	MT085360
9	IBV/EGY/CH/F742/2019	4/2019	Gharbia	MN651568	MT085350	MT085357
10	IBV/EGY/CH/F859/2019	8/2019	Sharqia	MN651569	MT085351	MT085355

Table 2. Primers sequences of S, N, and M genes of IBV

Gene	Primer sequence		Amplicon size	Reference
NP	3' NP-F 3'NP-IR	ATTCCAAGGGAAAACTTGTG TCCTCATTCATCTTGTCATCACC	832	The present study
	NP-IF NP-R	GGTATAGTGTGGGTTGCTG AGCTGTGCATTGTTCCTCTC	832	The present study
M	3'M-F 3'M-R	TTTTGGTATACATGGGTAG TACTCTCTACACACACACAT	880	The present study
S1	IBV-S1- F2 IBV- HVR3- Reverse	GATTGTGCATGGTGGACAATG CAGAYTGCTTRCAACCACC	1100	Abdel-moneim et al. (2002); Naguib et al. (2017)
	IBV-HVR3- Forward IBV-Oligo -3-Reverse	TACTGGTAATTTTTCAGATGG CATAACTAACATAAGGGCAA	900	Adzhar et al. (1997); Gelb et al. (2005)

Genetic and phylogenetic analysis

Nucleotide and amino acid sequences of ten isolates were alignments with other IBV strains representing different groups (classical, variant I, variant II) and vaccine seeds (H120, M41, Ma5, 4/91, CR88121, and D274) that were used in Egypt were obtained from the National Center for Biotechnology Information. The alignment was carried out with the CLUSTAL-W program and the MegAlign module of DNASTAR software (Lasergene version 7.2; DNASTAR, Madison, WI, USA). The phylogenetic tree was constructed using MEGA version 6 (Tamura et al., 2013) according to the maximum likelihood tree method with moderate strength and 1000 bootstrap replicates (Kumar et al., 2016). The pairwise nucleotide percent identity was calculated using DNA star software (DNAStar, Madison, WI). The glycosylation sites were detected using NetN-Glyc 1.0 Server (Gupta et al., 2004).

Estimation of selection pressure

The sequence of S gene from the ten isolated IBV was analyzed to determine the selection pressure for each gene segment by estimating the ratio of non-synonymous (dN) to synonymous (dS) substitutions ($\omega = dN/dS$) across the lineages towards a codon-by-codon basis. The selective pressure was defined as $\omega = 1$ indicates a neutral evolution, $\omega < 1$ denotes a negative or purifying selective pressure, and $\omega > 1$ refers to a positive selective pressure. The mean values of ω were calculated using the SLAC and FEL methods on the Datamonkey website (Delport et al., 2010).

The Recombination Detection Program (RDP 4, Version 4.95) was used to identify possible recombination events of S1, M, and N genes (Mo et al., 2013), including the algorithms RDP, Bootscan, Geneconv, MaxChi, Chimaera, SiScan, and 3Seq (Martin et al., 2015).

RESULTS

Genetic characterization of the spike gene

Phylogenetic analysis of the spike gene revealed that the ten Egyptian strains were clustered in GI 23 (variant II). The findings indicated that seven out of ten strains were clustered into Egy Var II, divided into two sub-groups (I, II) as shown in Figure 1. The spike gene had specific features, compared to the reference strain IBV/CU/4/2014 isolated from Egypt in 2014. It had 40 Amino Acid (AA) mutations with the exception of IBV/EG/CV32/ 2019 that had a specific character with 50 AA mutations in different sites. The other three strains were clustered to Egy Var I with specific features. The three strains had 17 AA mutations with the exception of IBV/EG/F859/ 2019 that had 15 AA in comparison with the reference strain IBV/CU/4/2014 isolated from Egypt in 2014.

Hypervariable Regions (HVRs) in the S1 gene demonstrated different patterns among different viruses, compared to the IBV/CU/4/2014 strains. All of the Egy Var II related strains in the new cluster had two, four, and eight AA mutations with the exception of IBV/EG/cv32/ 2019 with 6, 7, 8 AA mutations in the HVRI, II, III, respectively (Figure 2).

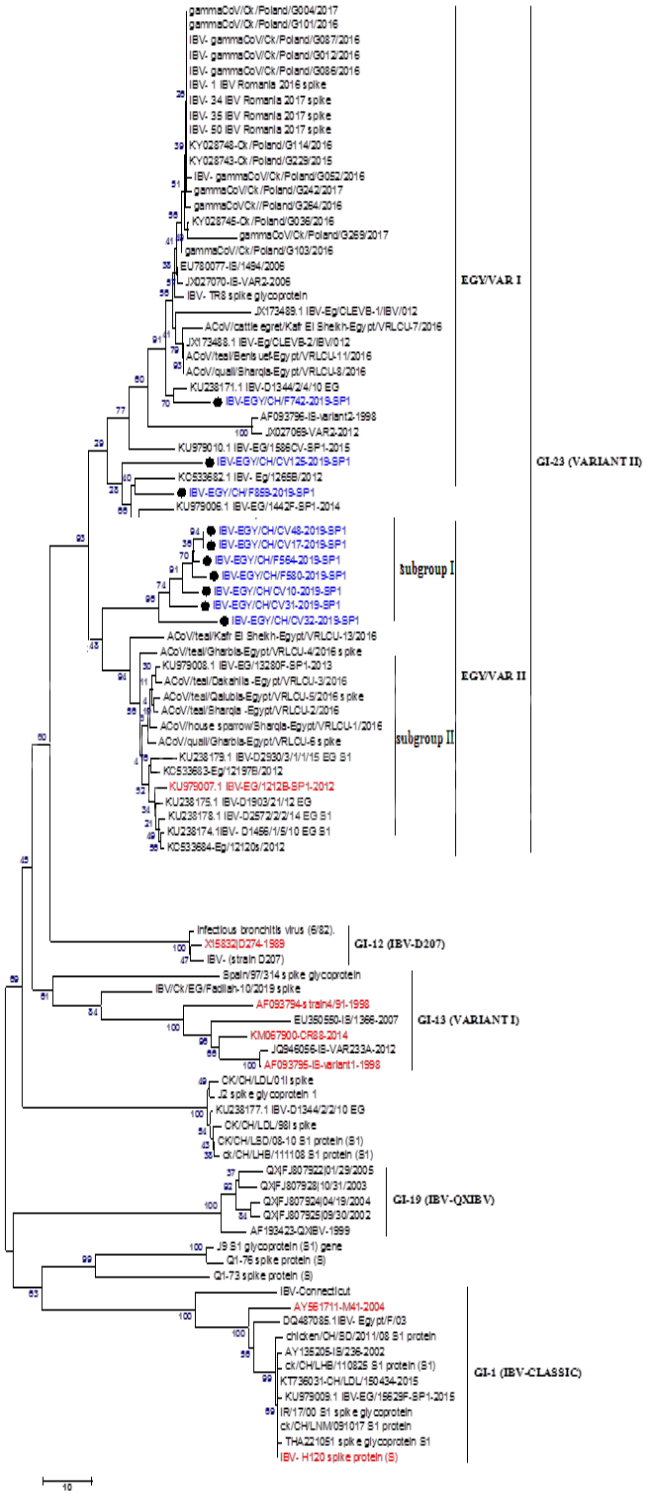


Figure 1. Phylogenetic tree of the S1 gene. Figure shows the phylogenetic analysis of the S1 gene indicating all Egyptian strain clusters into genotype GI-23 (variant II) with three strains were sub-clustered into Egy VAR I and the other seven isolates sub-clusters into Egy VAR II, dividing it into two subgroups (I, II). Black dots indicate viruses sequenced in the current study.

The other three strains related to Egy Var I had two AA mutations in the HVRI with the exception of IBV/EG/F859/ 2019 which had one AA mutation. The four AA mutations were detected in the HVRII, except that IBV/EG/F742/ 2019 with three AA mutations. Finally, HVRIII of IBV/EG/CV125/ 2019, IBV/EG/F859/ 2019, IBV/EG/F742/ 2019 had five, seven, and one AA mutations, respectively (Figure 2).

All of the IBV strains in the present study had 17 N-linked glycosylation sites. However, the isolated strains lacked the glycosylation site at position 139 and had an additional glycosylation site at AA 280, compared to IBV/CU/4/2014 reference strain with the exception of IBV/EG/F742/2019 and IBV/EG/CV32/2019, which had an additional one at only 280. Selection pressure analysis demonstrated five positive selections at position 53, 69,128, 232, and 262 in all strains of the S1 gene.

Phylogenetic analysis and amino acids identity of the S1 gene revealed that the variant IBV isolates (Egy/Var I and Egy/Var II) had a significant relationship with vaccine seeds commonly used in Egypt, including H120, M41, 4/91, CR88, D274, Var I 1/96, in the range of 69% to 83% (Table 3).

Genetic characterization of N gene

Phylogenetic analysis of the N gene classified IBV strains into classical and variant groups. The variant group was classified into variant I and variant II. The Egyptian strains in the present study were clustered in GI 23 variant II and divided into two sub-groups (I, II; Figure 3). All strains had specific features (16 AA mutations), compared to the reference strain IBV/CU/4/2014 isolated from Egypt in 2014. However, the IBV/EG/CV32/ 2019 had a specific character with 11 AA mutations at different sites with no selection pressure. The amino acid identity of the N gene of the 10 strains, compared to IBV vaccine seeds Egypt, including Ma5, H120, 4/91, CR88, ranged 91-95% (Supplementry Table 1). All strains presented one N-glycosylation site at N protein residue 32 (Site of glycosylation: NASW).

Genetic characterization of M gene

The phylogenetic analysis of the M gene revealed no differentiation between variant I and II. All isolated strains in the present study were clustered in the variant group and appeared in a new sub-group (Figure 4). All of the strains had nine AA mutations, compared to the reference strain IBV/CU/4/2014 isolated from Egypt in 2014 without any selection pressure. The nucleotide identity of the M gene of the ten strains, compared to IBV vaccine seeds IBV/H120, Ma41, 4/91, was within the range of 94-96% (Supplemntry Table 2). All strains showed only one N-glycosylation site at residue 3/4/6 (Site of glycosylation: NCTL).

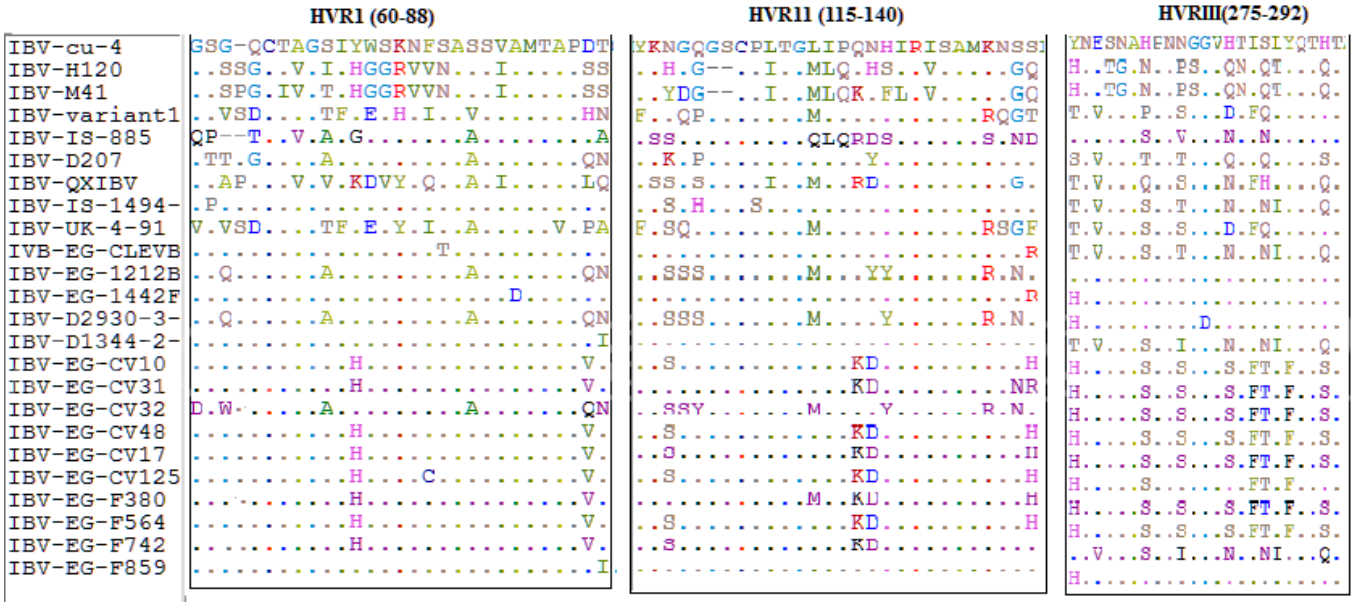


Figure 2. Hyper-variable regions of the S1 gene of IBV. The amino acid alignment and mutation of hyper-variable regions of tested isolates, compared to IBV/CU/4/2014.

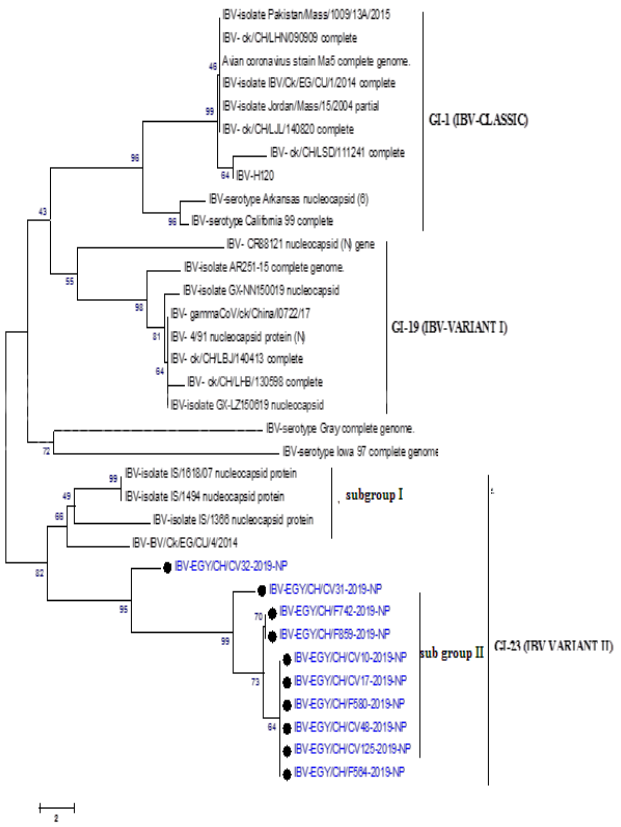


Figure 3. Phylogenetic tree of the N gene. The phylogenetic analysis of the N gene revealed that all Egyptian strain clusters into genotype GI-23 (variant II) divided it into two subgroups (I, II). Black dots indicate viruses that were sequenced in the current study.

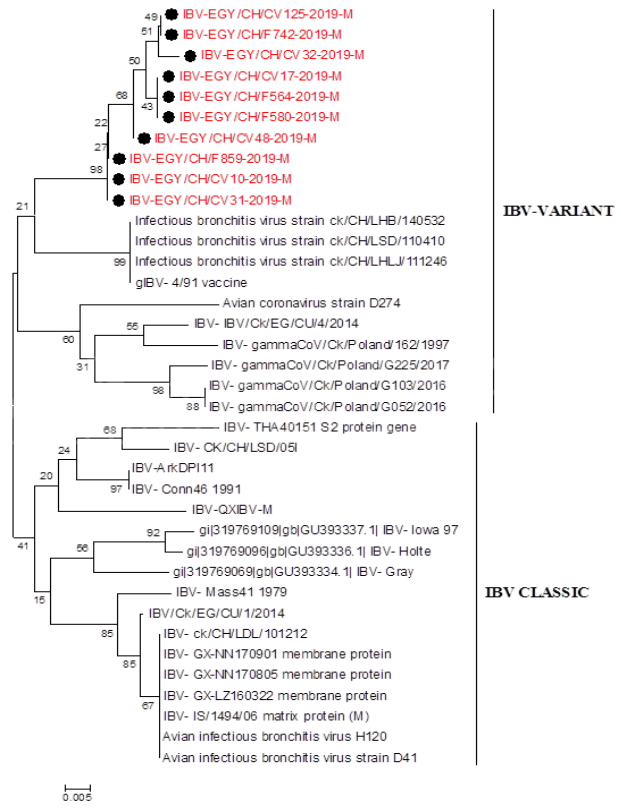


Figure 4. Phylogenetic tree of M gene. The phylogenetic analysis of the M gene revealed that all Egyptian strain clusters into the variant group in a new subgroup. Black dots indicate viruses that were sequenced in the current study.

Table 3. Nucleotide and Amino acid identities and divergence of S1 gene sequenced viruses compared to other selected strains and vaccine strains. Comparative alignment of the S1 gene showed that S1 A.A identity percent of tested strain ranged 69% to 83% with different vaccine seeds used in Egypt.

Sequence name	Ck/EG/CU/4/2014	EU780077-IS/1494/2006	IS/885-2003	D274-1989	AY561711-M41-2004	IBV-EG/1212B-SP1-2012	IBV-Eg/CLEVB-2/IBV/012	IS-variant1-1/96	IS-VAR2-2006	QXIBV-1999	IBV-Connecticut	IBV- (strain D207)	IBV- H120	CR88121-2014	AF093794-strain4/91-1998	IBV-EGY/CH/CV10-2019-SP1	IBV-EGY/CH/CV31-2019-SP1	IBV-EGY/CH/CV32-2019-SP1	IBV-EGY/CH/CV48-2019-SP1	IBV-EGY/CH/CV17-2019-SP1	IBV-EGY/CH/CV125-2019-SP1	IBV-EGY/CH/F580-2019-SP1	IBV-EGY/CH/F564-2019-SP1	IBV-EGY/CH/F742-2019-SP1	IBV-EGY/CH/F859-2019-SP1
	NUCLEOTIDE IDENTITY %																								
1. Ck/EG/CU/4/2014		95%	88%	83%	80%	95%	95%	79%	95%	78%	79%	83%	80%	79%	79%	93%	90%	84%	93%	93%	93%	93%	90%	94%	97%
2. IS/1494/2006	94%		88%	83%	80%	91%	99%	79%	100%	78%	79%	83%	80%	79%	80%	93%	88%	84%	93%	93%	91%	92%	90%	98%	94%
3. IS/885-2003	88%	88%		80%	79%	88%	88%	78%	88%	78%	78%	80%	79%	78%	79%	87%	83%	80%	87%	87%	85%	87%	84%	88%	88%
4. D274-1989	83%	84%	80%		80%	84%	83%	79%	83%	78%	79%	98%	80%	79%	79%	82%	79%	78%	82%	82%	79%	82%	79%	82%	82%
5. M41-2004	76%	75%	74%	77%		79%	80%	78%	80%	78%	95%	80%	97%	78%	78%	79%	76%	73%	79%	79%	76%	79%	76%	79%	79%
6. IBV-EG/1212B-SP1-2012	94%	90%	87%	84%	75%		91%	79%	91%	78%	78%	84%	79%	78%	78%	90%	88%	87%	89%	89%	89%	90%	86%	91%	95%
7. IBV-Eg/CLEVB-2/IBV/012	94%	99%	87%	83%	75%	90%		80%	99%	78%	79%	83%	80%	79%	80%	93%	88%	84%	93%	93%	91%	92%	90%	98%	94%
8. IS-variant1-1/96	79%	79%	77%	78%	74%	78%	79%		79%	78%	77%	79%	77%	97%	96%	78%	75%	73%	78%	78%	76%	79%	76%	78%	78%
9. IS-VAR2-2006	94%	100%	87%	83%	74%	90%	98%	79%		78%	79%	83%	80%	79%	79%	93%	88%	84%	93%	93%	91%	92%	90%	98%	94%
10. QXIBV-1999	78%	79%	79%	78%	77%	78%	79%	78%	78%		78%	78%	79%	79%	80%	78%	75%	73%	77%	78%	75%	77%	75%	78%	77%
11. IBV-Connecticut	76%	75%	75%	78%	91%	75%	75%	74%	75%	76%		79%	94%	77%	78%	78%	75%	73%	78%	78%	75%	78%	75%	78%	78%
12. IBV- (strain D207)	83%	83%	79%	98%	76%	84%	83%	78%	83%	78%	77%		80%	79%	79%	82%	79%	78%	82%	82%	79%	82%	79%	82%	82%
13. IBV- H120	77%	75%	76%	77%	96%	76%	76%	74%	75%	78%	91%	78%		77%	78%	79%	76%	73%	79%	79%	76%	79%	76%	79%	79%
14. CR88121-2014	79%	80%	76%	79%	74%	77%	80%	96%	79%	79%	74%	79%	74%		97%	78%	75%	73%	78%	78%	76%	79%	76%	78%	78%
15. AF093794-strain4/91-1998	78%	79%	78%	79%	74%	77%	79%	93%	79%	80%	74%	78%	75%	94%		78%	76%	73%	79%	79%	76%	79%	76%	79%	78%
16. IBV-EGY/CH/CV10-2019-SP1	92%	91%	86%	82%	74%	89%	91%	78%	91%	78%	74%	82%	74%	78%	78%		95%	89%	99%	99%	92%	99%	96%	94%	93%
17. IBV-EGY/CH/CV31-2019-SP1	88%	87%	83%	79%	72%	87%	87%	75%	86%	76%	72%	79%	73%	76%	76%	94%		93%	94%	94%	94%	94%	98%	90%	91%
18. IBV-EGY/CH/CV32-2019-SP1	84%	82%	80%	77%	69%	86%	82%	72%	82%	73%	70%	76%	69%	73%	73%	89%	92%		88%	89%	87%	89%	92%	84%	85%
19. IBV-EGY/CH/CV48-2019-SP1	91%	91%	86%	81%	74%	89%	91%	78%	91%	78%	74%	81%	74%	79%	78%	99%	94%	88%		100%	92%	99%	96%	94%	92%
20. IBV-EGY/CH/CV17-2019-SP1	91%	91%	86%	81%	74%	89%	91%	78%	91%	78%	74%	81%	74%	79%	78%	99%	94%	88%	100%		92%	99%	96%	94%	92%
21. IBV-EGY/CH/CV125-2019-SP1	91%	89%	84%	79%	72%	88%	89%	75%	89%	76%	72%	79%	73%	75%	75%	91%	93%	86%	92%	92%		91%	94%	92%	93%
22. IBV-EGY/CH/F580-2019-SP1	91%	90%	86%	81%	74%	89%	91%	79%	90%	78%	74%	81%	75%	79%	79%	98%	94%	88%	99%	99%	91%		96%	93%	92%
23. IBV-EGY/CH/F564-2019-SP1	88%	88%	82%	78%	71%	85%	87%	75%	87%	76%	72%	78%	72%	76%	76%	95%	97%	91%	96%	96%	94%	96%		91%	89%
24. IBV-EGY/CH/F742-2019-SP1	92%	96%	87%	82%	74%	89%	96%	78%	96%	78%	74%	82%	75%	79%	78%	92%	88%	82%	93%	93%	91%	92%	89%		94%
25. IBV-EGY/CH/F859-2019-SP1	97%	94%	87%	83%	75%	94%	94%	78%	93%	78%	75%	83%	76%	78%	78%	92%	90%	84%	91%	91%	92%	91%	88%	93%	
	AMINO ACIDS IDENTITY %																								

To cite this paper: Yehia N, Said D and Zanaty AM (2020). Characterization and Analysis of the Major Structural Protein Genes of the Recently Isolated Avian Infectious Bronchitis Virus in Egypt. *J. World Poult. Res.*, 10 (4): 649-661. DOI: https://dx.doi.org/10.36380/jwpr.2020.74

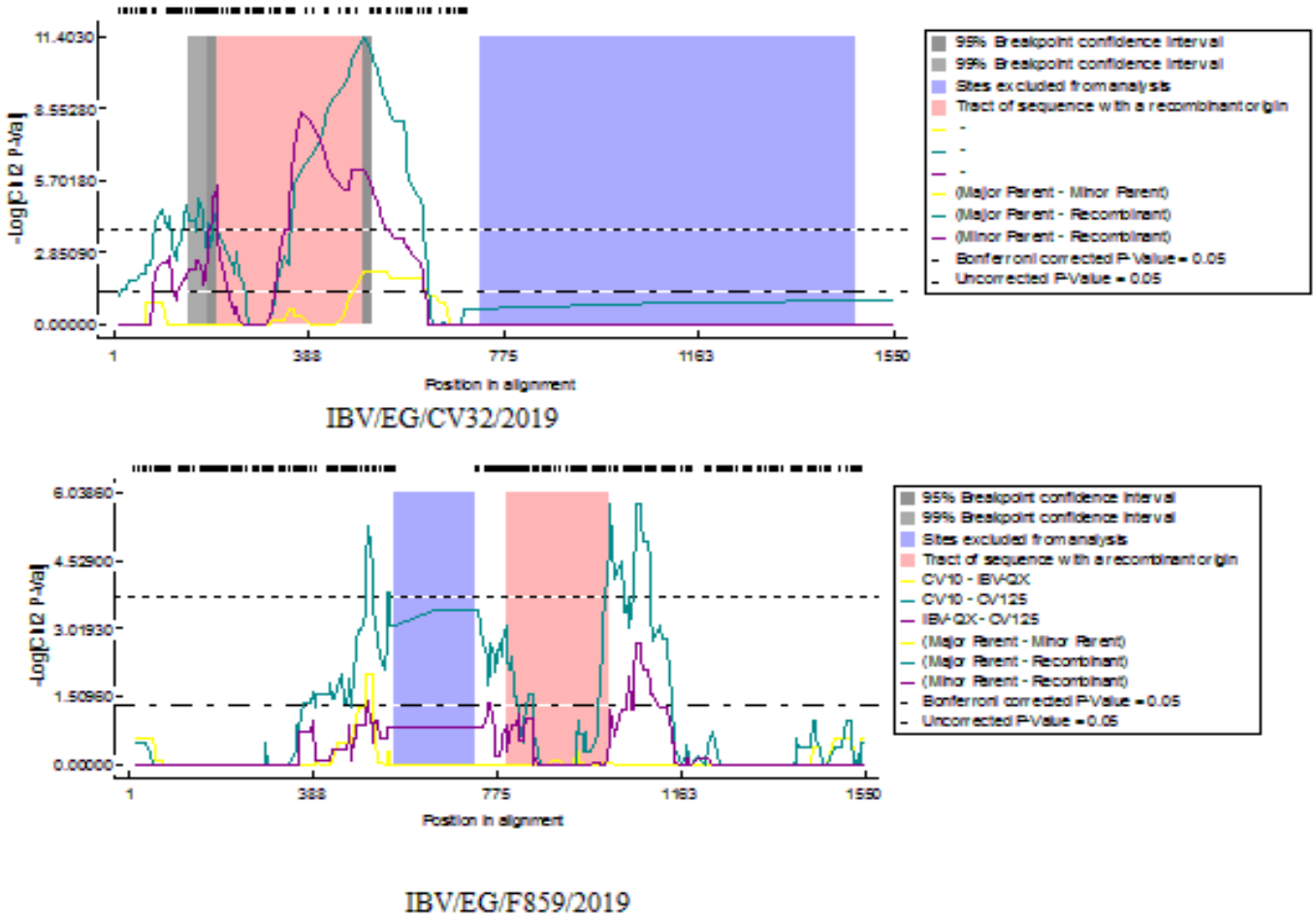


Figure 5. Recombination detection analysis of the S1 gene. Recombination events predicted for IBV/EG/CV32/2019 had a minor recombination from D274 and a major recombination from the Egyptian strain IBV/EG/F859 / 2019. However, the IBV/EG/F859/2019 had a minor recombination of QX and a major one of IBV/EG/CV10/2019.

Recombination analysis

The recombination events of the S1 were detected in two strains, with IBV/EG/CV32 / 2019 indicating a slight recombination from D274 and a larger recombination from the Egyptian strain IBV/EG/F859 / 2019. However, the IBV/EG/F859 / 2019 had a minor recombination of QX and a larger recombination of IBV/EG/CV10 / 2019 (Figure 5). No recombination events were recorded in the nucleotide sequences of the N and M genes.

DISCUSSION

The Infectious bronchitis virus (IBV) is still widespread worldwide and causes massive damage in the poultry industry in both vaccinated and non-vaccinated flocks (Lyb, 2010). Different studies have focused on the epidemiology of the virus (Fathy et al., 2014; Sultanet al., 2019). There is a study emphasizing the hypervariable region of the spike gene (HVR-S) (Abdel-Moneim et al., 2012; Zanaty et al., 2016), but limited genetic information about the major structural protein was reported (S, N, and M). The molecular characterization of S1 and N genes was responsible for the evolutionary analysis of IBV (Lee et al., 2003). Besides, the protein encoded by the S1 and N genes is the most potent antigens for inducing an immune response to IBV infection (Ignjatovic and Galli, 1995). The current study examined the genetic variability and recombination of IBV of the major structural protein (S1, N, and M).

Previous research suggested the genetic classification of IBV based on S1 HVR I (Lee et al., 2003; Zantay et al., 2016), but the findings were not representative due to the presence of multiple mutations throughout the S1 gene detected in the presented study and other previous studies (Schikora et al., 2003; Li et al., 2012). The S1 genes of all strains were clustered in GI-23

(variant II) with three strains clustered into Egy VAR I and the other seven strains clustered in Egy VAR II, as previously reported (Zanaty et al., 2016, Abozeid et al., 2017). However, all of the strains in the present study related to Egy VAR II became a new subgroup.

Multiple outbreaks in the presence of different vaccination programs were previously studied (Abd El Rahman et al., 2015; Sultan et al., 2013). The massive use of classic H120, M41, and variant 4/91 vaccines produced vaccination pressure on the virus leading to the production of a virus escape mutant in the HVR, and accordingly vaccination failure as reported in previous studies (Zanaty et al., 2016; Sultan et al., 2019). Different mutations in the HVRI, II, and III were detected in all strains possibly due to vaccination pressure. Moreover, the currently administered vaccine showed genetically different values as was mentioned before (Rohaim et al., 2019). In this regard, there is a need to conduct further studies to demonstrate the antigenicity ,pathogenicity and the effectiveness of the vaccine of recent field strains.

The N-glycosylation sites in the spike and membrane glycoproteins of IBV had a significant effect on the antigenicity, receptor binding and fusion (Braakman and Van Anken, 2000; Wissink et al., 2004). Variation in N-glycosylation sites could affect receptor interaction, reduce recognition of antibodies leading to a reduction in the innate immune response, and affect the replication and infectivity of the virus (Slater-Handshy et al., 2004; Vigerust and Shepherd, 2007). The difference in the N-glycosylation sites on the spike protein reported in current study requires further studies to show its effect on the pathogenicity of the virus. The N protein played an important role in immunogenicity against IBV infection, and the assembly of viruses (He et al., 2004; You et al., 2007). However, previous studies suggested that the N gene was conserved, which was supported by the detection of all IBV strains (Williams et al., 1992). all strains in this study were divided into a new subgroup with multiple specific mutations as well as the S1 gene with one N-glycosylation site in the N gene, as previously described (Fan et al., 2019). It is therefore required to investigate the effect of this mutation on the immunogenicity and pathogenicity of the virus.

The M protein is responsible for the assembly of virus particle by interactions with other structural proteins (Vennema et al., 1996). The phylogenetic analysis revealed that genotypes based on the S1 gene differ significantly from those of the M gene. There is no differentiation between variant I and II in the characterization of the M gene, as previously described (Shieh et al., 2004; Hughes, 2011). All strain clustered in the variant group with a new subgroup as well as the S1 gene. The rise of multiple new IBV genotypes was observed due to the occurrence of several recombination events within the same genotype or between different genotypes. Others were observed between field and vaccine viruses (Zhang et al., 2010; Han et al., 2016; Jackwood et al., 2010). The recombination was detected in the present study in two isolates from QX and D274 and the same genotype as previously detected in a study conducted by Kiss et al (2016) and no recombination was detected in the N or M gene.

Natural selection usually led to a reduction in harmful mutations, thus promoting beneficial mutations. In general, the gene positively selected by natural selection usually had very important functions (Tang et al., 2009). The positive selection pressure in this study was only detected at five sites in the S1 gene, and was expected due to the extensive use of IBV vaccine as previously described (Jahantigh et al., 2013). This selective pressure could affect the primary and secondary structures of the S1 gene, which led to a change in the genetic and molecular characterization of the virus and the emergence of new strains that, as previously reported, could escape from the immune system (Dolz et al., 2008). Therefore, more research is needed to determine the role of these mutations in the virulence of IBV.

CONCLUSION

The Egyptian IBV has evolved continuously and has acquired special features. The S1 protein is clustered to clad GI23 variant II (the genetic classification of IBV) with three strain clusters into Egy VARI and others cluster to Egy VARII in new subgroup, compared to the previously isolated strain in Egypt with specific mutations, especially in the HVRI, II, and III. The strains included in the study differed significantly from vaccine seeds. The molecular characterization of the M gene and N gene are confirmed as the classification of the S1 gene with a specific feature. The recombination detected in the present study occurred in two isolates from QX and D274. Surveillance of IBV should continue to ensure the early detection of virus mutations and to study the pathogenicity and antigenicity, as well as the evaluation of the vaccine efficacy against newly evolved strains.

DECLERATION

Author`s contribution

Nahed Yehia suggested the title of study and designed the paper, Dalia Said isolated the IBV samples.

Nahed Yehia and Ali Zanaty identified the molecular characterization of isolates. All authors participated in the writing, analysis of the data, and review of the manuscript, and finally approved the last version of manuscript.

Acknowledgement

This study was funded by Animal health research institute.

Competing interests

All authors declared that did not have any conflict of interest.

REFERENCES

Abd El Rahman S, Hoffmann M, Lueschow D, Eladl A, and Hafez HM (2015). Isolation and characterization of new variant strains of infectious bronchitis virus in Northern Egypt. Advanced in animal and veterinary science, 3: 326-471. DOI: http://dx.doi.org/10.14737/journal.aavs/2015/3.7.362.371

Abdel-Moneim AS, Afifi MA, and El-Kady MF (2012). Emergence of a novel genotype of avian infectious bronchitis virus in Egypt. Archive Virology, 157: 2453-2457. DOI: https://www.doi.org/10.1007/s00705-012-1445-1

Abdel-Moneim AS, Madbouly HM, Gelb Jr J, and Ladman BS (2002). Isolation and identification of Egypt/Beni-Suef/01 a novel genotype of infectious bronchitis virues. Veterinary Medicine Journal, Giza, 50: 1065-1078.

Abozeid H, Paldurai A, Khattar SK, Afifi MA, El-Kady MF, El-Deeb AH, and Samal SK (2017). Complete genome sequences of two avian infectious bronchitis viruses isolated in Egypt: Evidence for genetic drift and genetic recombination in the circulating viruses. Infection, Genetics and Evolution, 53: 7-14. Available at: https://pubmed.ncbi.nlm.nih.gov/28495648/

Adzhar A, Gough R, Haydon Daniel, Shaw K, Britton Paul, and Cavanagh D (1997). Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Avian pathology, 26: 625-40. DOI: https://www.doi.org/10.1080/03079459708419239

Ammayappan A, Upadhyay C, Gelb J, and Vakharia VN (2009). Identification of sequence changes responsible for the attenuation of avian infectious bronchitis virus strain Arkansas DP. Archieve Virology, 154: 495-499. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7086983/

Braakman I, and Van Anken E (2000). Folding of viral envelope glycoproteins in the endoplasmic reticulum. Traffic, 1(7): 533-539. DOI: https://www.doi.org/10.1034/j.1600-0854.2000.010702.x

Brierley I, Digard P, and Inglis SC (1989). Characterization of an efficient coronavirus ribosomal frame shifting signal: requirement for an RNA pseudoknot, Cell, 57: 537-547. Available at: https://pubmed.ncbi.nlm.nih.gov/2720781/

Carstens E (2009). Ratification vote on taxonomic proposals to the international committee on taxonomy of viruses. Archive Virology, 155: 133-146. Available at: https://pubmed.ncbi.nlm.nih.gov/27424026/

Cavanagh D, Casais R, Armesto M, Hodgson T, Izadkhasti S, Davies M, Lin F, Tarpey I, and Britton P (2007). Manipulation of the infectious bronchitis coronavirus genome for vaccine development and analysis of the accessory proteins. Vaccine, 25: 5558-5562. Available at: https://pubmed.ncbi.nlm.nih.gov/17416443/

Cavanagh D, Davis PJ, Cook JK, Li D, Kant A, and Koch G (1992). Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathololgy, 21: 33-43. Available at: https://pubmed.ncbi.nlm.nih.gov/18670913/

Corse E, and Machamer CE (2003). The cytoplasmic tails of infectious bronchitis virus E and M proteins mediate their interaction. Virology, 312: 25-34. Available at: https://pubmed.ncbi.nlm.nih.gov/12890618/

Delport W, Poon AF, Frost SD, and Kosakovsky Pond SL (2010). Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics, 26: 2455-2457. Available at: https://pubmed.ncbi.nlm.nih.gov/20671151/

Dolz R, Pujols J, Ordonez G, Porta R, and Majo N (2008). Molecular epidemiology and evolution of avian infectious bronchitis virus in Spain over a fourteen-year period. Virology, 374: 50-59. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7103278/

Fan W, Tang N, Dong Z, Chen J, Zhang W, Zhao C, He Y, Li M, Wu C, Wei T et al. (2019). Genetic Analysis of Avian Coronavirus Infectious Bronchitis Virus in Yellow Chickens in Southern China over the Past Decade: Revealing the Changes of Genetic Diversity, Dominant Genotypes, and Selection Pressure. Viruses, 11: 898. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6833030/

Fathy RR, El boraay, IM, El shorbagy MA, and El-Mahdy SS (2014). A survey on presence of new strains of infectious bronchitis virus in some chicken farms of Egyptian Delta provinces during. Benha veterinary medical journal, 2: 248-262. Available at: https://bvmj.journals.ekb.eg/article_32511.html

Gelb JJ, Weisman Y, Ladman B, and Meir R (2005). Gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996-2000). Avian Pathology, 34: 194-203. Available at: https://pubmed.ncbi.nlm.nih.gov/16191702/

Gupta R, Jung E, and Brunak S (2004). Prediction of N-Glycosylation Sites in Human Proteins. 46: 203-206. Available at: https://pubmed.ncbi.nlm.nih.gov/11928486/

Han Z, Zhang T, Xu Q, Gao M, Chen Y, Wang Q, Zhao Y, Shao Y, Li H, and Kong X et al. (2016). Altered pathogenicity of a tl/CH/LDT3/03 genotype infectious bronchitis coronavirus due to natural recombination in the 5'-17 kb region of the genome. Virus Research, 213: 140-148. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7114521/

Hassan MSH., Ojkic D, Coffin CS, Cork SC, van der Meer F, Abdul-Careem MF (2019). Delmarva (DMV/1639) Infectious Bronchitis Virus (IBV) variants isolated in eastern Canada show evidence of recombination. Viruses, 11: 1054. Available at: https://pubmed.ncbi.nlm.nih.gov/31766215/

He R, Dobie F, Ballantine M, Leeson A, Li Y, Bastien N, Cutts T, Andonov A, Cao J, Booth TF et al. (2004). Analysis of multimerization of the SARS coronavirus nucleocapsid protein. Biochemical Biophysical Research Community, 316: 476-483. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7111152/

Hewson KA, Noormohammadi AH, Devlin JM, Browning GF, Schultz BK, and Ignjatovic J (2014). Evaluation of a novel strain of infectious bronchitis virus emerged as a result of spike gene recombination between two highly diverged parent strains. Avian Pathology, 43: 249-257. Available at: https://www.tandfonline.com/doi/full/10.1080/03079457.2014.914624

Hughes AL (2011). Recombinational histories of avian infectious bronchitis virus and turkey coronavirus. Archive Virology, 156: 1823–1829. DOI: https://www.doi.org/10.1007/s00705-011-1061-5

Ignjatovic J, and Galli U (1995). Immune responses to structural proteins of avian infectious bronchitis virus. Avian Pathology, 24(2): 313-332. Available at: https://pubmed.ncbi.nlm.nih.gov/18645789/

Jackwood MW (2012). Review of infectious bronchitis virus around the world. Avian Disease, 56: 634-641. Available at: https://pubmed.ncbi.nlm.nih.gov/23397833/

Jackwood MW, Boynton TO, Hilt DA, McKinley ET, Kissinger JC, Paterson AH, Robertson J, Lemke C, McCall AW, and Williams SM et al. (2010). Emergence of a group 3 coronavirus through recombination. Virology, 398: 98-108. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7111905/

Jahantigh M, Salari S, and Hedayati M (2013). Detection of infectious bronchitis virus serotypes by reverse transcription polymerase chain reaction in broiler chickens. Springer plus, 2(1): 36. Available at: https://springerplus.springeropen.com/articles/10.1186/2193-1801-2-36

Kiss I, Mató T, and Homonnay Z (2016). Successive occurrence of recombinant infectious bronchitis virus strains in restricted area of Middle East. Virus Evulsion, 2: vew021. Available at: https://pubmed.ncbi.nlm.nih.gov/29492274/

Kumar S, Stecher G, and Tamura K (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular and Biological evolution, 33: 1870-1874. Available at: https://pubmed.ncbi.nlm.nih.gov/27004904/

Lai MMC, and Holmes KV (2001). Coronaviridae: the viruses and their replication. In: Knipe, Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4369385/

Lee CW, Hilt DA, and Jackwood MW (2003). Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. Journal of veterinary Diagnostic Investigation, 15: 344-348. Available at: https://pubmed.ncbi.nlm.nih.gov/12918815/

Li M, Wang XY, Wei P, Chen QY, Wei ZJ, and Mo ML (2012). Serotype and genotype diversity of infectious bronchitis viruses isolated during 1985–2008 in Guangxi, China. Archive Virology, 157: 467-474.

Lyb V (2010). Diagnosis of infectious bronchitis: an overview of concepts and tools Revista Brasileira de Ciência Avícola .Brazilain journal of poultry science, 12: 10. Available at: https://www.scielo.br/scielo.php?pid=S1516-635X2010000200006&script=sci_abstract

Martin DP, Murrell B, Golden M, Khoosal A, and Muhire B (2015). RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evolution, 1. Available at: https://academic.oup.com/ve/article/1/1/vev003/2568683

Masters P, and Perlman S (2013). Coronaviridae in Fields of virology, Lippincot Williams and Wilkins, Philadelphia, USA. Available at: https://www.umassmed.edu/globalassets/ambros-lab/meetings/rna-biology-club-2019_20/masters-and-perlman-2013-in-fields-virology_1.pdf

Milek J, and Blicharz-Domanska K (2018). Coronaviruses in avian species-review with focus on epidemiology and diagnosis in wild birds. Journal of Veterinary. Research, 62: 249-255. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6296008/

Mo ML, Li M, Huang BC, Fan WS, Wei P, Wei TC, Cheng QY, Wei ZJ, and Lang YH (2013). Molecular characterization of major structural protein genes of avian coronavirus infectious bronchitis virus isolates in southern China. Viruses, 5: 3007-3020.

Naguib MM, El-Kady MF, Lüschow D, Hassan KE, Arafa AS, El-Zanaty A, Hassan MK, Hafez HM, Grund C, and Harder TC (2017). New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle disease viruses. Journal of virological methods, 245: 19-27. Available at: https://www.sciencedirect.com/science/article/abs/pii/S0166093416302105

Rohaim MA, El Naggar RF, Hamoud MM, Bazid AI, Gamal AM, Laban SE, Abdel-Sabour MA, Nasr SAE, Zaki MM, Shabbir MZ et al. (2019). Emergence and genetic analysis of variant pathogenic 4/91 (serotype 793/B) infectious bronchitis virus in Egypt during, 55: 720-725. Available at: https://pubmed.ncbi.nlm.nih.gov/31372921/

Schikora BM, Shih LM, and Hietala SK (2003). Genetic diversity of avian infectious bronchitis virus California variants isolated between 1988 and 2001 based on the S1 subunit of the spike glycoprotein. Archive Virology, 148: 115-136. Available at: https://link.springer.com/article/10.1007/s00705-002-0904-5

Shieh HK, Shien JH, Chou HY, Shimizu Y, Chen JN, and Chang PC (2004). Complete nucleotide sequences of S1 and N genes of infectious bronchitis virus isolated in Japan and Taiwan. Journal of veterinary medicine science, 66: 555-558. Available at: https://pubmed.ncbi.nlm.nih.gov/15187369/

Slater-Handshy T, Droll DA, Fan X, Di Bisceglie AM, and Chambers TJ (2004). HCV E2 glycoprotein: mutagenesis of N-linked glycosylation sites and its effects on E2 expression and processing. Virology, 319: 36-48. Available at: https://www.sciencedirect.com/science/article/pii/S0042682203007736

Sultan H, Abdel-Razik AG, Shehata AA, Ibrahim M, Talaat S,Abo-Elkhair M, Bazid AE, Moharam IM, and Vahlenkamp T (2013). Characterization of Infectious Bronchitis Viruses Circulating in Egyptian Chickens during 2012 and 2013. Journal of Veterinary Science & Medical Diagnosis, 4: 5. Available at: https://www.scitechnol.com/peer-review/characterization-of-infectiousbronchitis-viruses-circulating-inegyptian-chickens-during-2012and-2013-tfHD.php?article_id=4042

Sultan HA, Ali A, El Feil WK, Bazid AHI, Zain El-Abideen MA, and Kilany WH (2019). Protective efficacy of different live attenuated infectious bronchitis virus vaccination regimes against challenge with IBV variant-2 circulating in the Middle East. Frontiers in Veterinary Science, 6: 341. Available at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00341/full

Tamura K, G Stecher, D Peterson, A Filipski, and S Kumar (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology evolution, 30: 2725-2729.Avilable at: https://pubmed.ncbi.nlm.nih.gov/24132122/

Tang X, Li G, Vasilakis N, Zhang Y, Shi Z, Zhong Y, Wang LF, and Zhang S (2009). Differential stepwise evolution of SARS coronavirus functional proteins in different host species. Evolutionary Biology, 9: 52. Available at: https://bmcevolbiol.biomedcentral.com/articles/10.1186/1471-2148-9-52

Thor SW, Hilt DA, Kissinger JC, Paterson AH, and Jackwood MW (2011). Recombination in avian gamma-coronavirus infectious bronchitis virus. Viruses, 3: 1777-1799. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3187689/

Valastro V, Holmes EC, Britton P, Fusaro A, Jackwood MW, Cattoli G, and Monne I (2016). S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. Infectious genetic evolution, 39: 349-364. Available at: https://www.sciencedirect.com/science/article/abs/pii/S1567134816300466

Vennema H, Godeke GJ, Rossen JW, Voorhout WF, Horzinek MC, Opstelten DJ, and Rottier PJ (1996). Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. The EMBO Journal, 15: 2020-2028. Available at: https://pubmed.ncbi.nlm.nih.gov/8617249/

Vigerust DJ, and Shepherd VL (2007). Virus glycosylation: role in virulence and immune interactions. Trends Microbiology, 15(5): 211-218. Available at: https://pubmed.ncbi.nlm.nih.gov/17398101/

Wang C, Zheng X, Gai W, Zhao Y, Wang H, Wang H, Feng N, Chi H, Qiu B, Li N et al. (2017). MERS-CoV virus-like particles produced in insect cells induce specific humeral and cellular immunity in

rhesus macaques. Oncotarget, 8(8): 12686-12694. Available at: https://pubmed.ncbi.nlm.nih.gov/27050368/

Williams A, Wang L, Sneed L, and Collisson E (1992). Analysis of a hypervariable region in the 3' non-coding end of the infectious bronchitis virus genome. Virus Research, 25: 213-222. Available at: https://www.sciencedirect.com/science/article/abs/pii/0168170293900863

Wissink EH, Kroese MV, Maneschijn-Bonsing JG, Meulenberg JJ, Van Rijn PA, Rijsewijk FA, and Rottier PJ (2004). Significance of the oligosaccharides of the porcine reproductive and respiratory syndrome virus glycoproteins GP2a and GP5 for infectious virus production. The Journal of General Virology, 85(12): 3715-3723. Available at: https://pubmed.ncbi.nlm.nih.gov/15557245/

You JH, Reed ML, and Hiscox JA (2007). Trafficking motifs in the SARS coronavirus nucleocapsid protein. Biochemical Biophysical Research Communication, 358: 1015-1020. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7092899/

Zanaty A, Arafa AS, Hagag N, and El-Kady M (2016). Genotyping and pathotyping of diversified strains of infectious bronchitis viruses circulating in Egypt. World journal of virology, 5(3): 125-134. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4981825/

Zhang Y, Wang HN, Wang T, Fan WQ, Zhang AY, Wei K, Tian GB, and Yang X (2010). Complete genome sequence and recombination analysis of infectious bronchitis virus attenuated vaccine strain H120. Virus Genes, 41: 377-388. Available at: https://pubmed.ncbi.nlm.nih.gov/20652731/

Supplementary Table 1. Nucleotide identities and divergence of N gene sequenced viruses compared to other selected strains and vaccine strains. The comparative alignment of N gene showed that the percentage of N AA identity of the tested strain ranged 91- 95% with different vaccine seeds used in Egypt.

Sequence name	IBV/Ck/EG/CU/4/2014	IBV-isolate AR251-15	IBV- 4/91	IBV- CR88121	IBV-serotype Arkansas	IBV/Ck/EG/CU/1/2014,	Avian coronavirus strain Ma5,	IBV-isolate IS/1494	IBV-H120	IBV-EGY/CH/CV10-2019-NP	IBV-EGY/CH/CV17-2019-NP	IBV-EGY/CH/CV31-2019-NP	IBV-EGY/CH/F580-2019-NP	IBV-EGY/CH/CV48-2019-NP	IBV-EGY/CH/CV125-2019-NP	IBV-EGY/CH/F564-2019-NP	IBV-EGY/CH/CV32-2019-NP	IBV-EGY/CH/F742-2019-NP	IBV-EGY/CH/F859-2019-NP
	Nucleotide Identity %																		
1. IBV-IBV/Ck/EG/CU/4/2014		91%	92%	92%	92%	92%	92%	98%	92%	94%	94%	93%	94%	94%	94%	94%	95%	95%	95%
2. IBV-isolate AR251-15	95%		99%	92%	91%	91%	91%	91%	91%	90%	90%	91%	90%	90%	90%	90%	93%	91%	91%
3. IBV- 4/91	96%	99%		93%	92%	92%	92%	92%	92%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
4. IBV- CR88121	95%	96%	96%		93%	92%	92%	92%	92%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
5. IBV-serotype Arkansas	95%	95%	95%	95%		97%	97%	92%	97%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
6. IBV/Ck/EG/CU/1/2014,	94%	95%	96%	94%	98%		100%	93%	100%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
7. Avian coronavirus Ma5,	94%	95%	96%	94%	98%	100%		93%	100%	91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
8. IBV-isolate IS/1494	98%	96%	95%	96%	95%	95%	95%		93%	94%	94%	93%	94%	94%	94%	94%	95%	94%	94%
9. IBV-H120	94%	95%	95%	94%	97%	100%	100%	95%		91%	91%	91%	91%	91%	91%	91%	93%	91%	91%
10. IBV-EGY/CH/CV10-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%		100%	99%	100%	100%	100%	100%	96%	99%	99%
11. IBV-EGY/CH/CV17-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%		99%	100%	100%	100%	100%	96%	99%	99%
12. IBV-EGY/CH/CV31-2019-NP	95%	94%	94%	94%	93%	92%	92%	95%	92%	99%	99%		99%	99%	99%	99%	95%	99%	99%
13. IBV-EGY/CH/F580-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%	100%	99%		100%	100%	100%	96%	99%	99%
14. IBV-EGY/CH/CV48-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%	100%	99%	100%		100%	100%	96%	99%	99%
15. IBV-EGY/CH/CV125-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%	100%	99%	100%	100%		100%	96%	99%	99%
16. IBV-EGY/CH/F564-2019-NP	96%	93%	93%	94%	92%	92%	92%	96%	91%	100%	100%	99%	100%	100%	100%		96%	99%	99%
17. IBV-EGY/CH/CV32-2019-NP	97%	95%	95%	95%	94%	94%	94%	97%	94%	97%	97%	97%	97%	97%	97%	97%		96%	96%
18. IBV-EGY/CH/F742-2019-NP	96%	94%	93%	93%	92%	92%	92%	96%	92%	100%	100%	99%	100%	100%	100%	100%	98%		100%
19. IBV-EGY/CH/F859-2019-NP	96%	94%	93%	93%	92%	92%	92%	96%	92%	100%	100%	99%	100%	100%	100%	100%	98%	100%	
	Amino Acids Identity %																		

To cite this paper: Yehia N, Said D and Zanaty AM (2020). Characterization and Analysis of the Major Structural Protein Genes of the Recently Isolated Avian Infectious Bronchitis Virus in Egypt. *J. World Poult. Res.*, 10 (4): 649-661. DOI: https://dx.doi.org/10.36380/jwpr.2020.74

Supplementary table 2. Nucleotide identities and divergence of M gene sequenced viruses compared to other selected strains and vaccine strains. The comparative alignment of M gene showed that the percentage of M gene AA identity of the tested strain ranged 94-96% with different vaccine seeds used in Egypt.

Sequence name	IBV/Ck/EG/CU/4/2014,	IBV-QXIBV-M	IBV/Ck/EG/CU/1/2014	IBV- M41,	IBV-Arkansas Vaccine,	IBV- Conn46 1996,	IBV- 4/91 vaccine,	IBV-AR251-15	IBV-IS/1494/06	IBV-D41	IBV-H120	IBV-EGY/CH/CV32-2019-M	IBV-EGY/CH/CV31-2019-M	IBV-EGY/CH/CV125-2019-M	IBV-EGY/CH/F859-2019-M	IBV-EGY/CH/CV48-2019-M	IBV-EGY/CH/F742-2019-M	IBV-EGY/CH/CV17-2019-M	IBV-EGY/CH/CV10-2019-M	IBV-EGY/CH/F564-2019-M	IBV-EGY/CH/F580-2019-M
Nucleotide Identity %																					
1. IBV/Ck/EG/CU/4/2014,		92%	93%	93%	90%	90%	92%	95%	92%	93%	93%	96%	96%	96%	96%	96%	96%	96%	96%	96%	96%
2. IBV-QXIBV-M	93%		91%	91%	91%	91%	92%	94%	90%	90%	90%	92%	92%	92%	91%	92%	92%	92%	91%	92%	92%
3. IBV/Ck/EG/CU/1/2014	94%	95%		97%	93%	93%	94%	93%	99%	100%	100%	93%	94%	94%	93%	94%	93%	94%	93%	93%	93%
4. IBV- M41,	94%	95%	100%		92%	92%	93%	93%	96%	96%	96%	92%	93%	93%	93%	93%	92%	93%	92%	93%	93%
5. IBV-Arkansas Vaccine,	93%	95%	96%	96%		100%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	91%	92%
6. IBV- Conn46 1996,	93%	95%	96%	96%	100%		92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	92%	91%	92%
7. IBV- 4/91 vaccine,	94%	94%	95%	95%	95%	95%		94%	94%	94%	94%	93%	93%	93%	93%	93%	93%	93%	93%	93%	93%
8. IBV-AR251-15.	95%	94%	95%	95%	95%	95%	95%		93%	93%	93%	95%	95%	95%	95%	95%	95%	95%	95%	95%	95%
9. IBV-IS/1494/06	93%	94%	99%	99%	95%	95%	94%	94%		99%	99%	92%	93%	93%	93%	93%	92%	93%	93%	93%	93%
10. IBV-D41	94%	94%	100%	100%	95%	95%	95%	94%	100%		100%	93%	93%	93%	93%	93%	93%	93%	93%	93%	93%
11. IBV-H120	94%	94%	100%	100%	95%	95%	95%	94%	100%	100%		93%	93%	93%	93%	93%	93%	93%	93%	93%	93%
12. IBV-EGY/CH/CV32-2019-M	96%	94%	94%	94%	94%	94%	95%	96%	94%	94%	94%		99%	99%	99%	99%	100%	99%	100%	99%	99%
13. IBV-EGY/CH/CV31-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%		100%	100%	100%	99%	100%	99%	100%	100%
14. IBV-EGY/CH/CV125-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%	100%		100%	100%	99%	100%	99%	100%	100%
15. IBV-EGY/CH/F859-2019-M	95%	94%	94%	94%	94%	94%	95%	95%	93%	94%	94%	99%	99%	99%		100%	99%	100%	99%	100%	100%
16. IBV-EGY/CH/CV48-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%	100%	100%	99%		99%	100%	99%	100%	100%
17. IBV-EGY/CH/F742-2019-M	96%	94%	94%	94%	94%	94%	95%	96%	94%	94%	94%	100%	100%	100%	99%	100%		99%	100%	99%	99%
18. IBV-EGY/CH/CV17-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%	100%	100%	99%	100%	99%		99%	100%	100%
19. IBV-EGY/CH/CV10-2019-M	96%	94%	94%	94%	94%	94%	95%	96%	94%	94%	94%	100%	100%	100%	99%	100%	100%	99%		99%	100%
20. IBV-EGY/CH/F564-2019-M	95%	94%	94%	94%	94%	94%	95%	95%	93%	94%	94%	99%	99%	99%	100%	99%	99%	100%	99%		99%
21. IBV-EGY/CH/F580-2019-M	96%	94%	95%	95%	94%	94%	96%	96%	94%	94%	94%	100%	100%	100%	99%	100%	99%	100%	100%	99%	
	Amino Acids Identity %																				

Instructions for Authors

JWPR EndNote Style
Manuscript Template (MS Word)
Sample Articles
Declaration form
Policies and Publication Ethics

Manuscript as Original Research Paper, Short Communication, Case Reports and Review or Mini-Review are invited for rapid peer-review publishing in *the Journal of World's Poultry Research*. Considered subject areas include: Husbandry and management; construction, environment and welfare; exotic and wild birds; Biochemistry and cellular biology; immunology, avian disease control; layer and quail management; nutrition and feeding; physiology, genetics, reproduction and hatching; technology, processing and food safety... view full aims and scope

Submission

The manuscript and other correspondence should preferentially be submit online. Please embed all figures and tables in the manuscript to become one single file for submission. Once submission is complete, the system will generate a manuscript ID and will send an email regarding your submission. Meanwhile, the authors can submit or track articles via editor [at] jwpr.science-line.com or editorjwpr [at] gmail.com. All manuscripts must be checked (by English native speaker) and submitted in English for evaluation (in totally confidential and impartial way).

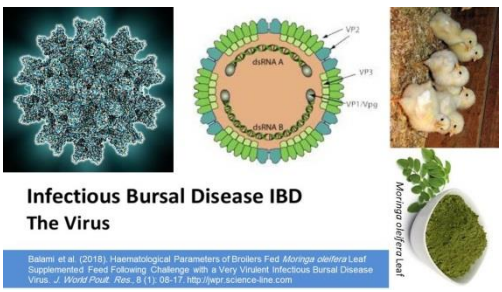
Supplementary information:
The online submission form allows supplementary information to be submitted together with the main manuscript file and covering letter. If you have more than one supplementary files, you can submit the extra ones by email after the initial submission. Author guidelines are specific for each journal. Our Word template can assist you by modifying your page layout, text formatting, headings, title page, image placement, and citations/references such that they agree with the guidelines of journal. If you believe your article is fully edited per journal style, please use our MS Word template before submission.

Supplementary materials may include figures, tables, methods, videos, and other materials. They are available online linked to the original published article. Supplementary tables and figures should be labeled with a "S", e.g. "Table S1" and "Figure S1". The maximum file size for supplementary materials is 10MB each. Please keep the files as small possible to avoid the frustrations experienced by readers with downloading large files.

Submission to the Journal is on the understanding that:
1.The article has not been previously published in any other form and is not under consideration for publication elsewhere;
2.All authors have approved the submission and have obtained permission for publish work.
3.Researchers have proper regard for conservation and animal welfare considerations. Attention is drawn to the *'Guidelines for the Treatment of Animals in Research and Teaching*'. Any possible adverse consequences of the work for populations or individual organisms must be weighed against the possible gains in knowledge and its practical applications. If the approval of an ethics committee is required, please provide the name of the committee and the approval number obtained.

Ethics Committee Approval
Experimental research involving animals should have been approved by author's institutional review board or ethics committee. This information can be mentioned in the manuscript including the name of the board/committee that gave the approval. The use of animals in experiments will have observed the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education by the New York Academy of Sciences, Ad Hoc Animal Research Committee.

Graphical Abstract
Authors should provide a graphical abstract (a beautifully designed feature figure) to represent the paper aiming to catch the attention and interest of readers. Graphical abstract will be published online in the table of content. The graphical abstract should be colored, and kept within an area of 12 cm (width) x 6 cm (height) or with similar format. Image should have a minimum resolution of 300 dpi and line art 1200dpi.
Note: Height of the image should be no more than the width. Please avoid putting too much information into the graphical abstract as it occupies only a small space. Authors can provide the graphical abstract in the format of PDF, Word, PowerPoint, jpg, or png, after a manuscript is accepted for publication. For preparing a Professional Graphical Abstract, please click **here**.



Presentation of the article

Main Format

First page of the manuscripts must be properly identified by the title and the name(s) of the author(s). It should be typed in Times New Roman (font sizes: 17pt in capitalization for the title, 10pt for the section headings in the body of the text and the main text, double spaced, in A4 format with 2cm margins. All pages and lines of the main text should be numbered consecutively throughout the manuscript. Abbreviations in the article title are not allowed.
Manuscripts should be arranged in the following order:
1. TITLE (brief, attractive and targeted);

2. Name(s) and Affiliation(s) of author(s) (including post code) and corresponding E-mail; ORCID: 0000-0000-0000-0000
3. ABSTRACT
4. Key words (separate by semicolons; or comma,)
5. Abbreviations (used in the manuscript)
6. INTRODUCTION
7. MATERIALS AND METHODS
8. RESULTS
9. DISCUSSION
10. CONCLUSION
11. DECLARATIONS
12. REFERENCES
13. Tables
14. Figure captions
15. Figures

Results and Discussion can be presented jointly.
Discussion and Conclusion can be presented jointly.

Article Sections Format

Title should be a brief phrase describing the contents of the paper. The first letter of each word in title should use upper case. The Title Page should include the author(s)'s full names and affiliations, the name of the corresponding author along with phone and e-mail information. Present address (es) of author(s) should appear as a footnote.

Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The abstract should be 150 to 350 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.

Following the abstract, about 3 to 8 key words that will provide indexing references should be listed.

Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and Methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail. The ethical approval for using animals in the researches should be indicated in this section with a separated title.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the author(s)'s experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the results but should be put into the discussion section.

Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

Conclusion should be brief and tight about the importance of the work or suggest the potential applications and extensions. This section should not be similar to the Abstract content.

Declarations including Ethics, Consent to publish, Competing interests, Authors' contributions, and Availability of data and materials are necessary.

Acknowledgments of persons, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph forms or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or PowerPoint before pasting in the Microsoft Word manuscript file. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

Declarations section - Please include declarations heading

Please ensure that the sections:
-Ethics (and consent to participate)
-Consent to publish
-Competing interests
-Authors' contributions
-Availability of data and materials
are included at the end of your manuscript in a Declarations section.

Consent to Publish
Please include a 'Consent for publication' section in your manuscript. If your manuscript contains any individual person's data in any form (including individual details, images or videos), consent to publish must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent to publish. You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication). If your manuscript does not contain any individual persons data, please state "Not applicable" in this section.

Authors' Contributions
For manuscripts with more than one author, JWPR require an Authors' Contributions section to be placed after the Competing Interests section.
An 'author' is generally considered to be someone who has made substantive intellectual contributions to a published study. To qualify as an author one should 1) have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) have been involved in drafting the manuscript or revising it critically for important intellectual content; and 3) have given final approval of the version to be published. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.
We suggest the following format (please use initials to refer to each author's contribution): AB carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. JY carried out the immunoassays. MT participated in the sequence alignment. ES participated in the design of the study and performed the statistical analysis. FG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.
For authors that equally participated in a study please write 'All/Both authors contributed equally to this work.' Contributors who do not meet the criteria for authorship should be listed in an acknowledgements section.

Competing Interests
Competing interests that might interfere with the objective presentation of the research findings contained in the manuscript should be declared in a paragraph heading "Competing interests" (after Acknowledgment section and before References). Examples of competing interests are ownership of stock in a company, commercial grants, board membership, etc. If there is no competing interest, please use the statement "The authors declare that they have no competing interests.".
Journal World's Poultry Research adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE authorship criteria should be based on 1) substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data, 2) drafting the article or revising it critically for important intellectual content and 3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3. It is a requirement that all authors have been accredited as appropriate upon submission of the manuscript. Contributors who do not qualify as authors should be mentioned under Acknowledgements.

Change in authorship
We do not allow any change in authorship after provisional acceptance. We cannot allow any addition, deletion or change in sequence of author name. We have this policy to prevent the fraud.

Acknowledgements
We strongly encourage you to include an Acknowledgements section between the Authors' contributions section and Reference list. Please acknowledge anyone who contributed towards the study by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data, or who was involved in drafting the manuscript or revising it critically for important intellectual content, but who does not meet the criteria for authorship. Please also include their source(s) of funding. Please also acknowledge anyone who contributed materials essential for the study.
Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements. Please list the source(s) of funding for the study, for each author, and for the manuscript preparation in the acknowledgements section. Authors must describe the role of the funding body, if any, in study design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Data Deposition
Nucleic acid sequences, protein sequences, and atomic coordinates should be deposited in an appropriate database in time for the accession number to be included in the published article. In computational studies where the sequence information is unacceptable for inclusion in databases because of lack of experimental validation, the sequences must be published as an additional file with the article.

References:

A JWPR reference style for **EndNote** may be found **here**.

1. All references to publications made in the text should be presented in a list with their full bibliographical description. DOI number or the link of article should be added to the end of the each reference.
2. In the text, a reference identified by means of an author's name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author's surname should be mentioned, followed by 'et al'. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like 'a' and 'b' after the date to distinguish the works.
3. References in the text should be arranged chronologically (e.g. Kelebeni, 1983; Usman and Smith, 1992 and Agindotan et al., 2003). The list of references should be arranged alphabetically on author's surnames, and chronologically per author. If an author's name in the list is also mentioned with co-authors, the following order should be used: Publications of the single author, arranged according to publication dates - publications of the same author with one co-author - publications of the author with more than one co-author. Publications by the same author(s) in the same year should be listed as 1992a, l992b, etc.
4. Names of authors and title of journals, published in non-latin alphabets should be transliterated in English.
5. A sample of standard reference is "1th Author surname A, 2th Author surname B and 3th Author surname C (2013). Article title should be regular and 9 pt. Journal of World`s Poultry Research, Volume No. (Issue No.): 00-00." DOI:XXX."
6. Journal titles should be full in references. The titles should not be italic.
7. References with more than 10 authors should list the first 10 authors followed by 'et al.'
8. The color of references in the text of article is blue. Example: (Preziosi et al., 2002; Mills et al., 2015).

9. At least 35% of the references of any submitted manuscript (for all types of article) should include scientific results published in the last five years.

-Examples (at the text- blue highlighted)

Abayomi (2000), Agindotan et al. (2003), Vahdatpour and Babazadeh (2016), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; Chukwura, 1987a,b; Tijani, 1993, 1995), (Kumasi et al., 2001).

--Examples (at References section)

a) For journal:

Lucy MC (2000). Regulation of ovarian follicular growth by somatotropin and insulin- like growth factors in cattle. Journal of Dairy Science, 83: 1635-1647.

Kareem SK (2001). Response of albino rats to dietary level of mango cake. Journal of Agricultural Research and Development. pp 31-38. DOI:XXX.

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. African Journal of Biotechnology, 7: 3535-3539. DOI:XXX.

Tahir Khan M, Bhutto ZA, Abbas Raza SH, Saeed M, Arain MA, Arif M, Fazlani SA, Ishfaq M, Siyal FA, Jalili M et al. (2016). Supplementation of different level of deep stacked broiler litter as a source of total mixed ration on digestibility in sheep and their effects on growth performance. Journal of World`s Poultry Research, 6(2): 73-83. DOI: XXX

b) For symposia reports and abstracts:

Cruz EM, Almatar S, Aludul EK and Al-Yaqout A (2000). Preliminary Studies on the Performance and Feeding Behaviour of Silver Pomfret (Pampus argentens euphrasen) Fingerlings fed with Commercial Feed and Reared in Fibreglass Tanks. Asian Fisheries Society Manila, Philippine 13: 191-199.

c) For edited symposia, special issues, etc., published in a journal:

Korevaar H (1992). The nitrogen balance on intensive Dutch dairy farms: a review. In: A. A. Jongebreur et al. (Editors), Effects of Cattle and Pig Production Systems on the Environment: Livestock Production Science, 31: 17-27.

d) For books:

AOAC (1990). Association of Official Analytical Chemists. Official Methods of Analysis, 15th Edition. Washington D.C. pp. 69-88. Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

e) Books, containing sections written by different authors:

Kunev M (1979). Pig Fattening. In: A. Alexiev (Editor), Farm Animal Feeding. Vol. III. Feeding of Different Animal Species, Zemizdat, Sofia, p. 233-243 (Bg).

In referring to a personal communication the two words are followed by the year, e.g. (Brown, J. M., personal communication, 1982). In this case initials are given in the text.

Nomenclature and Abbreviations:

Nomenclature should follow that given in NCBI web page and Chemical Abstracts. Standard abbreviations are preferable. If a new abbreviation is used, it should be defined at its first usage. Abbreviations should be presented in one paragraph, in the format: "term: definition". Please separate the items by ";".
E.g. ANN: artificial neural network; CFS: closed form solution; ...

Abbreviations of units should conform with those shown below:

Decilitre	dl	**Kilogram**	kg
Milligram	mg	**hours**	h
Micrometer	mm	**Minutes**	min
Molar	mol/L	**Mililitre**	ml
Percent	%		

Other abbreviations and symbols should follow the recommendations on units, symbols and abbreviations: in "A guide for Biological and Medical Editors and Authors (the Royal Society of Medicine London 1977).
Papers that have not been published should be cited as "unpublished". Papers that have been accepted for publication, but not yet specified for an issue should be cited as "to be published". Papers that have been submitted for publication should be cited as "submitted for publication".

Formulae, numbers and symbols:

1. Typewritten formulae are preferred. Subscripts and superscripts are important. Check disparities between zero (0) and the letter 0, and between one (1) and the letter I.
2. Describe all symbols immediately after the equation in which they are first used.
3. For simple fractions, use the solidus (/), e.g. 10 /38.
4. Equations should be presented into parentheses on the right-hand side, in tandem.
5. Levels of statistical significance which can be used without further explanations are *P < 0.05, **P < 0.01, and ***P < 0.001
6. In the English articles, a decimal point should be used instead of a decimal comma.
7. In chemical formulae, valence of ions should be given, e.g. Ca2+ and CO32-, not as Ca++ or CO3.
8. Numbers up to 10 should be written in the text by words. Numbers above 1000 are recommended to be given as 10 powered x.
9. Greek letters should be explained in the margins with their names as follows: Aα - alpha, Bβ - beta, Γγ - gamma, Δδ - delta, Eε - epsilon, Zζ - zeta, Hη - eta, Θθ - theta, Iι - iota, Kκ - kappa, Λλ - lambda, Mμ - mu, Nν - nu, Ξξ - xi, Oo - omicron, Ππ - pi, Pρ - rho, Σσ - sigma, Tτ - tau, Yυ - ipsilon, Φφ - phi, Xχ - chi, Ψψ - psi, Ωω - omega.

Review/Decisions/Processing

Firstly, all manuscripts will be checked by Docol©c, a plagiarism finding tool. A single blind reviewing model is used by JWPR for non-plagiarized papers. The manuscript is edited and reviewed by the English language editor and three reviewers selected by section editor of JWPR respectively. Also, a reviewer result form is filled by reviewer to guide authors. Possible decisions are: accept as is, minor revision, major revision, or reject. See sample of evaluation form. Authors should submit back their revisions within 14 days in the case of minor revision, or 30 days in the case of major revision.

To submit a revision please click here, fill out the form, and mark "Revised", mention the article code (for example JWPR-1105), attach the revision (MS word) and continue submission. After review and editing the article, a final formatted proof is sent to the corresponding author once again to apply all suggested corrections during the article process. The editor who received the final revisions from the corresponding authors shall not be hold responsible for any mistakes shown in the final publication. Manuscripts with significant results are typically reviewed and published at the highest priority.

Plagiarism
There is a zero-tolerance policy towards plagiarism (including self-plagiarism) in our journals. Manuscripts are screened for plagiarism by Docol©c a plagiarism finding tool, before or during publication, and if found they will be rejected at any stage of processing. See sample of Docol©c-Report.

Declaration
After manuscript accepted for publication, a declaration form will be sent to the corresponding author who that is responsible to coauthors' agreements to publication of submitted work in JWPR after any amendments arising from the peer review.

Date of issue
The journal will be issued on 25th of March, June, September and December, each year.

Publication charges
No peer-reviewing charges are required. However, the publication costs are covered through article processing charges (APCs). There is a modest APC of 90 Euro(€) editor fee for the processing of each primary accepted paper (1000-4000 words) to encourage high-quality submissions. APCs are only charged for articles that pass the pre-publication checks and are published. A surcharge will be placed on any article that is over 4000 words in length to cover the considerable additional processing costs. Payment can be made by credit card, bank transfer, money order or check. Instruction for payment is sent during publication process as soon as manuscript is accepted. Meanwhile, this journal encourages the academic institutions in low-income countries to publish high quality scientific results, free of charges.

WORD COUNT	PRICE*
1000-4000 words (medium article)	€90
over 4000 words (long article)	€120

* The prices are valid until 30th January 2020.

The Waiver policy
The submission fee will be waived for invited authors, authors of hot papers, and corresponding authors who are editorial board members of the *Journal of World's Poultry Research*. The Journal will consider requests to waive the fee for cases of financial hardship (for high quality manuscripts and upon acceptance for publication). Requests for waiver of the submission fee must be submitted via individual cover letter by the corresponding author and cosigned by an appropriate institutional official to verify that no institutional or grant funds are available for the payment of the fee. Letters including the manuscript title and manuscript ID number should be sent to: editor [at] jwpr.science-line.com. It is expected that waiver requests will be processed and authors will be notified within two business day.

The OA policy
Journal of World's Poultry Research is an open access journal which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the *BOAI definition of Open Access*.

Scienceline Language Editing Services

We suggest that authors whose first language is not English have their manuscripts checked by a native English speaker before submission. This is optional, but will help to ensure that any submissions that reach peer review can be judged exclusively on academic merit. We offer a Scienceline service, and suggest that authors contact as appropriate. Please note that use of language editing services is voluntary, and at the author's own expense. Use of these services does not guarantee that the manuscript will be accepted for publication, nor does it restrict the author to submitting to Scienceline journals. You can send the article/s to the following Email: daryoushbabazadeh@gmail.com

Paper Submission Flow

Submission Preparation Checklist

Authors are required to check off their submission's compliance with all of the following items, and submissions may be returned to authors that do not adhere to the following guidelines.
The submission has not been previously published, nor is it before another journal for consideration (or an explanation has been provided in Comments to the Editor).
The submission file is in Microsoft Word, RTF, or PDF document file format.
Where available, URLs for the references have been provided.
The text is single-spaced; uses a 12-point font; and all illustrations, figures, and tables are placed within the text at the appropriate points, rather than at the end.
The text adheres to the stylistic and bibliographic requirements outlined in the Author Guidelines.

(Revised on 26 June 2019)

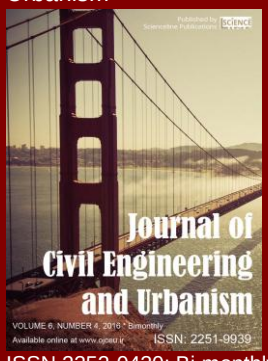
SCIENCELINE PUBLISHING CORPORATION

Scienceline Publication Ltd is a limited liability non-profit non-stock corporation incorporated in Turkey, and also is registered in Iran. Scienceline journals that concurrently belong to many societies, universities and research institutes, publishes internationally peer-reviewed open access articles and believe in sharing of new scientific knowledge and vital research in the fields of life and natural sciences, animal sciences, engineering, art, linguistic, management, social and economic sciences all over the world. Scienceline journals include:

Online Journal of Animal and Feed Research

ISSN 2228-7701; Bi-monthly
View Journal I Editorial Board
Email: editors@ojafr.ir
Submit Online >>

Journal of Civil Engineering and Urbanism



ISSN 2252-0430; Bi-monthly
View Journal I Editorial Board
Email: ojceu@ojceu.ir
Submit Online >>

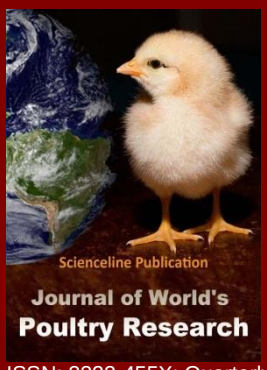
Journal of Life Sciences and Biomedicine

ISSN: 2251-9939; Bi-monthly
View Journal I Editorial Board
Email: editors@jlsb.science-line.com
Submit Online >>

Asian Journal of Medical and Pharmaceutical Researches

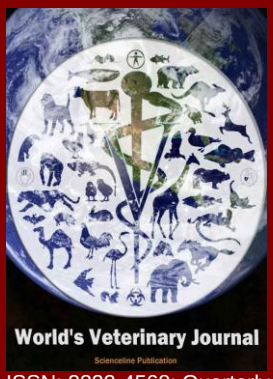
ISSN: 2322-4789; Quarterly
View Journal I Editorial Board
Email: editor@ajmpr.science-line.com
Submit Online >>

Journal of World's Poultry Research



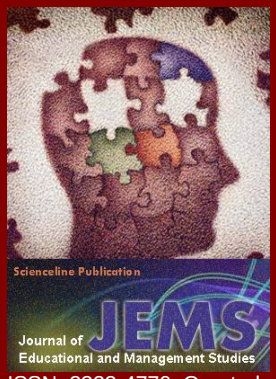
ISSN: 2322-455X; Quarterly
View Journal I Editorial Board
Email: editor@jwpr.science-line.com
Submit Online >>

World's Veterinary Journal



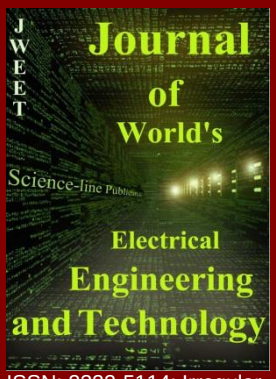
ISSN: 2322-4568; Quarterly
View Journal I Editorial Board
Email: editor@wvj.science-line.com
Submit Online >>

Journal of Educational and Management Studies



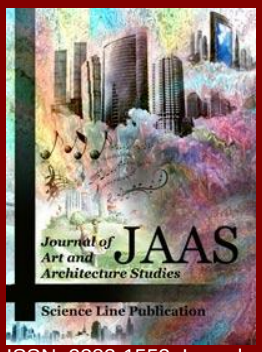
ISSN: 2322-4770; Quarterly
View Journal I Editorial Board
Email: info@jems.science-line.com
Submit Online >>

Journal of World's Electrical Engineering and Technology



ISSN: 2322-5114; Irregular
View Journal I Editorial Board
Email: editor@jweet.science-line.com
Submit Online >>

Journal of Art and Architecture Studies



ISSN: 2383-1553; Irregular
View Journal I Editorial Board
Email: jaas@science-line.com
Submit Online >>

Asian Journal of Social and Economic Sciences

ISSN: 2383-0948; Quarterly
View Journal I Editorial Board
Email: ajses@science-line.com
Submit Online >>

Journal of Applied Business and Finance Researches

ISSN: 2382-9907; Quarterly
View Journal I Editorial Board
Email: jabfr@science-line.com
Submit Online >>

Scientific Journal of Mechanical and Industrial Engineering

ISSN: 2383-0980; Quarterly
View Journal I Editorial Board
Email: sjmie@science-line.com
Submit Online >>